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Part I.

THE MORPHOLOGY, THE GROWTH AND THE DISSOCIATION OF *PASTEURELLA PESTIS*.

BY

MAJOR S. S. BHATNAGAR, M.D., Ph.D., F.R.C.P., I.M.S.

(*From the Central Research Institute, Kasauli.*)

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INTRODUCTION.

THE earlier investigations on *Pasteurella pestis* were undertaken at a time when the methods for the antigenic analysis of an organism and those for the study of its dissociation, as understood and practised at present, had not been developed.

The later studies where newer methods have been applied to the plague bacillus and the closely allied organism of pseudotuberculosis are comparatively few. Schütze (1932) showed that while *Pasteurella pestis* is possessed of only two antigens: (*a*) an envelope antigen in relation to the structure so described by Rowland (1914) and (*b*) a somatic antigen—*Pasteurella pseudotuberculosis*, on the other hand, contains three different antigens—a flagellar, a 'smooth' somatic and a 'rough' somatic. The serological relationship between these two organisms, according to this author, is due to the fact that the somatic antigen of the plague bacillus is identical with the 'rough' somatic antigen of the bacterium of pseudotuberculosis. This finding implies that *Pasteurella pestis* exists permanently in a 'rough' state. Schütze attributes an important rôle to the envelope antigen in plague immunity. Since this antigen has been shown by him to develop best when the temperature of incubation is 37°C., it would appear to follow that this should be the optimum temperature of incubation in the preparation of a plague vaccine. There are, however, other workers (Sokhey, 1932-35; Otten, 1935, 1935*a*) who do not agree with all these views.

The pioneer work of Haffkine resulted in the preparation of a plague prophylactic the main principles of which were the growth of a virulent strain of *Pasteurella*

pestis in broth at 27°C. for a few weeks and its subsequent sterilization by heat and by the addition of carbolic acid. From an account given by Taylor (1933) of the circumstances attending the first preparation and use of the vaccine by Haffkine, it will be evident that the selection of the method which Haffkine employed was largely on empirical grounds and that no real experimental basis existed on which the details of procedure could be founded. This method is still followed at the Haffkine Institute, Bombay, although modifications have been introduced in the original procedure by subsequent workers, specially by Sokhey (1939). It is claimed that the present Haffkine vaccine is of higher protective value than that made in earlier years. Taylor (*loc. cit.*), from an analysis of a large amount of available information on the results of inoculation with the Haffkine vaccine which, however, is admittedly of limited statistical value, worked out a figure of fourfold protection against attack and eightfold protection against death.

In the preparation of any plague prophylactic the employment of a virulent strain of *Pasteurella pestis* was considered by Haffkine and many other workers to be an essential factor. The recent studies of Otten (1936-37, 1938) in Java and that of Girard and Robic (1938) in Madagascar, on the other hand, indicate that the virulence of the organism *per se* does not play such an essential rôle in plague prophylaxis as has so far been suggested. Following the earlier experience of Strong (1906), these workers carried out mass inoculation of human beings with live avirulent suspensions of special strains of the plague bacillus. The statistical evidence from both these sources in conjunction with the laboratory experiments of Otten (1938) would suggest the superiority of this type of plague vaccine over a killed vaccine. Otten emphasizes the necessity of selecting only those cultures for the purpose of inoculation which he characterizes as 'smooth', as opposed to 'rough', according to his criteria. The latest developments in this connection are the findings of Schütze (1939) who, from a comparative study of various *Pasteurella pestis* antigens as prophylactic agents, concludes that a virulent strain possesses no superiority over an avirulent strain in this respect nor the 'smooth' organism over the 'rough' type of bacillus.

The wide divergence of opinion in regard to the most suitable method of producing an effective antiplague vaccine, involving such differences in practice as the use of killed or living suspensions, or of virulent or avirulent strains, indicates the necessity of determining what antigenic fractions in the organism are of essential importance in producing immunity. This requires to be studied in relation to the different types of *Pasteurella pestis* which have been used in the preparation of a vaccine, including both the virulent and the avirulent strains, under different conditions of maintenance and growth. With this object in view material was collected from as many sources as possible and the 92 strains of the plague bacillus thus obtained were divided into the following categories :—

Virulent strains .. { (*a*) Fully virulent.
(*b*) Partially virulent.
(*c*) Virulent.

Avirulent strains .. { (*d*) Protective avirulent.
(*e*) Non-protective avirulent.

The division of virulent strains into fully virulent and partially virulent was made possible through the courtesy of Lieut.-Colonel S. S. Sokhey, I.M.S., Director, Haffkine Institute, Bombay, who, before supplying us with these cultures, tested them according to the method developed by him (Sokhey, 1939). He employed the following criteria for his classification :—

(*a*) *Fully virulent.*—Fifty to 100 organisms per Haffkine Institute inbred mouse kill 100 per cent of the animals used and 5 to 10 organisms kill 80 per cent of the animals : death within about 7 days.

(*b*) *Partially virulent.*—More than 50 organisms (but less than 500) per Haffkine Institute inbred mouse kill 80 per cent of the animals used.

Strains received from other sources labelled 'virulent' were tested morphologically, biochemically and by a serological method to be described. It was not considered necessary to subject them to animal test for reasons which will be advanced later.

The avirulent strains were divided into two separate categories, viz., 'protective avirulents' and 'non-protective avirulents', since these presented important differences which will be referred to later. The protective avirulent group included (*a*) two well-known strains of Dr. Otten from Java, namely, (*i*) 'Tjiwidej', both its 'rough' and 'smooth' forms and (*ii*) 'Soemdoeng', and (*b*) three strains of Dr. Girard and Dr. Robic from Madagascar including their well-known strain 'E. V.' The non-protective avirulent group consisted of: (*a*) four strains from Bombay—'I', 'P', 'Q' and '120/5H'. These strains were originally virulent in character but they were attenuated by continuous subculturing on rabbit-blood agar at 37°C. for prolonged periods. The virulent variant of one of these and a partially virulent variant of another was also available, (*b*) and strain 'TRU' recently separated by Schütze (1939) from strain 'Tjiwidej rough' of Otten.

Conflicting opinions have been expressed by different workers as to the serological relationship of *Pasteurella pestis* and *Pasteurella pseudotuberculosis* (Zlatogorov, 1904 ; MacConkey, 1908 ; Albrecht, 1910 ; McCoy, 1911 ; Saisawa, 1913 ; Lerche, 1927 ; Arkwright, 1927 ; Zlatogorov and Mogilevskaia, 1928 ; Kauffmann, 1932). Since the collection of plague cultures was a very representative one it was decided to study this subject at the same time. Cultures of *Pasteurella pseudotuberculosis* belonging to group I, types A and B ; group II, types A and B ; and group III (Schütze, 1928) were obtained from the National Collection of Type Cultures, London, and were included in this study.

The various aspects of the bacteriology of *Pasteurella pestis* and of *Pasteurella pseudotuberculosis* which have been investigated are being communicated in four separate parts, namely, (*a*) morphology, growth and dissociation, (*b*) serology, (*c*) biochemical behaviour, and (*d*) immunological behaviour. The bearing of these findings on the problem of plague immunity will be discussed as a whole at the end of the last communication.

THE MORPHOLOGY, THE GROWTH AND THE DISSOCIATION OF *PASTEURELLA PESTIS.*

Along with the projected investigations into its antigenic structure it was considered desirable to study the biological characters and the metabolism of the plague bacillus under different conditions of growth and environment as these factors may presumably be related to antigenic differences in the various types of *Pasteurella pestis* strains.

Since evidence was available which suggested that the protective value of a plague prophylactic is influenced by the liquid or the solid nature of the substrate as well as by the temperature of incubation (Sokhey, 1932-35) both ordinary nutrient agar and ordinary nutrient broth were employed as the basal media. These were supplemented by the addition of accessory nutritive substances such as horse serum, carbohydrates and rabbit blood. A wide range of temperatures of incubation was adopted, namely, 37°C., 30°C., 27°C. and 22°C. room temperature and the temperature of the cold room (± 4°C.). During the period of this study the room temperature (Kasauli altitude 6,000 feet) varied from 18°C. to 20°C. The temperatures of the incubators were checked by placing maximum-minimum thermometers inside.

As a routine the representative members of the virulent and the avirulent strains were grown on the following media at the various temperatures referred to above :—

(1) Ordinary	Agar	and	broth
(2) Rabbit blood (5 per cent)	,,	,,	,,
(3) Horse serum (10 per cent)	,,	,,	,,
(4) Glucose (0·25 per cent, 0·5 per cent, 1 per cent)	,,	,,	,,
(5) Serum-glucose	,,	,,	,,

(*a*) *The morphology of* Pasteurella pestis.

The methods employed for the study of morphology were: (1) dark-ground examination, (2) staining by aniline dyes, (3) special capsular staining and (4) stained Indian ink preparations.

With regard to the comparative value of these methods it was our experience that examination by dark-ground illumination and staining either by aniline dyes or by special capsular stains did not reveal any information which could not be obtained by the study of stained Indian ink preparations alone. On the other hand, the first three methods presented a great drawback in that the structure described as the ' envelope ' of the plague bacillus by Rowland (*loc. cit.*) could not be demonstrated satisfactorily by any one of them. All that was visible of this was a halo round the organisms when grown at the higher temperatures—37°C. and 30°C.—especially on media to which serum and glucose had been added.

To determine whether the plague bacillus was possessed of a capsule, analogous to that of other organisms such as the *pneumococcus*, the technique recommended

by Churchman and Emelianoff (1933) and the well-known method of Muir were applied to both the virulent and the avirulent strains. Although a halo was seen to be present round organisms stained by these methods no evidence of the presence of a ' capsule ' could be obtained irrespective of the temperature of incubation and the medium of growth. The bacilli, however, stained better than with an ordinary aniline dye, such as carbol fuchsin, and their soma, both in the case of the virulent and the avirulent strains, was differentiated in some degree into an inner endoplasm and an outer ectoplasm. It is possible to mistake the ectoplasm for a capsule but the structure which has been designated as the ' envelope ' could not be demonstrated by this method and is obviously not of the same nature as the true capsule of other organisms.

When a suspension of the plague bacillus was mixed with Indian ink in the manner recommended by Rowland and by Schütze, a structure of the type described as the ' envelope ' by these authors was to be seen round a large majority of our *Pasteurella pestis* strains. For an extensive study in which records had to be maintained it was, however, considered that the method of these authors suffered from two disadvantages, namely, that (*a*) the preparations could not be preserved permanently, and (*b*) it was not possible to study the morphology of the organism at the same time so that separate staining for the body of the bacillus had to be resorted to. A method free from both these defects was, therefore, looked for. Butt, Bunynge and Joyce (1936) have described a technique for the study of the capsule round hæmolytic *streptococci*. This was adopted with modifications and was found to give very satisfactory results.

A method for the combined study of the envelope and the soma of the plague bacillus.—The slides were made free from grease by roasting them in a flame. A drop of 6 per cent dextrose solution was placed on one end of the slide and a thin even suspension made in it with the growth of the organism taken directly from an agar slope or broth. A drop of Indian ink was then poured on to the bacterial suspension and, with the aid of a spreader, a film was prepared in such a manner that it was thick in certain places and thin in others. This was allowed to dry and was then stained with a mixture of equal parts of methyl alcohol and one per cent methylene blue for a period of one hour. This procedure resulted in the organisms being stained greenish blue, standing out in marked contrast to the white envelope, both being clearly visible against the background of Indian ink (*see* Plate I).

All Indian inks do not give equally good results. Some of them have a tendency to deposit as small brownish black particles which make it difficult to study the morphology of the organisms. The best results were obtained by the use of ' Pelican ' waterproof drawing ink manufactured by Gunther-Wagner. Care should be taken to prevent any deposit of the stain on the slide. This is avoided if washing is undertaken by dipping the slide, with the stain still on top, in a bowl of running tap-water. The slide should then be left to dry naturally. It is necessary that the methylene blue should not be alkaline in reaction since the alkali tends to dissolve the envelope substance.

All the 92 strains collected by us were examined by this method. The findings can be discussed in relation to the origin and certain characters of the strains and in regard to the effect of the medium employed and the temperature of incubation.

In the bacterial cell itself different conditions of growth and of environment were seen to have brought about certain changes, which will be described later, but these were found to bear no relationship to the virulence or the avirulence of the organism, all the strains being affected equally. On the other hand, quantitative differences were noticed in the amount of the envelope substance possessed by different plague cultures. Roughly all the *Pasteurella pestis* strains could be divided into groups in which the envelope substance was (*a*) present in large quantities, (*b*) present to a limited degree and (*c*) not shown to be present by the method of examination employed.

All those cultures included under the heading of 'virulent strains' were possessed of the envelope substance in a fairly large quantity. Individual differences between the 'fully virulent' and 'partially virulent' organisms from this point of view could not be detected morphologically.

In the case of the strains to which the term 'protective avirulent' has been applied, the envelope substance was seen to be present but to a lesser degree when compared with the virulent strains. This group includes strains whose live suspensions have been employed for the purpose of mass human inoculation campaigns in Java and in Madagascar. The distinction between the virulent and the protective avirulent groups was, however, not so easy to establish morphologically. After incubation for 24 hours at the higher temperatures (37°C. and 30°C.) the organisms belonging to both these groups were seen to be possessed of a well-defined envelope. It was only when they were cultured on different media for a greater length of time that differences became evident on close examination. It was then observed that whereas the envelope round the protective avirulent strain was translucent in character that round the virulent strain was opaque. Secondly, the dye employed for staining the bacterial cell penetrated better through the envelope of the former type of organism with the result that its soma was better defined and possessed a deeper hue. With the virulent strain, on the other hand, in spite of one hour's staining, the bacilli could not be said to have taken the stain properly. This was particularly so if they had been grown on serum-glucose media and for periods longer than 24 hours. These observations suggested that there was a quantitative difference in the amount of the envelope substance which these two organisms were capable of producing. Confirmation in this connection was obtained by agglutination-absorption tests (*see* Part II of this paper).

The strains in which no evidence of the presence of an envelope could be obtained included four cultures from Bombay, namely, strains 'I', 'P', 'Q' and '120/5H' and strain 'TRU'. It has already been stated that the Bombay strains were originally virulent in character but had been attenuated by weekly subculture on rabbit-blood agar with incubation at 37°C., for prolonged periods. The fifth strain of this category—strain 'TRU'—had been recently separated by Schütze (1939) from Otten's strain 'Tjiwidej rough' and has been shown by him to possess

no envelope. All these strains were designated as 'non-protective avirulents' in view of their exhibiting practically no protective power in animal tests (Sokhey, 1936; Schütze, 1939).

A normal plague culture is characterized by its typical stickiness so that its growth can be drawn out in the form of a thread with the aid of a platinum loop. The non-protective avirulent strains did not show any such viscidity. On agar they grew in the form of what may be described as a continuous flake which cracked and left an irregular edge when a portion of the growth was picked up. A suspension of any one of them, either in water or in normal saline solution, presented the appearance which one associates with a 'rough' type of growth in the typhoid-paratyphoid group of organisms. The viscidity of a normal plague culture may presumably be due to the presence of the envelope substance.

The effect on the plague bacillus of prolonged subculturing on various media.—The representative strains of the three types of *Pasteurella pestis*—virulent, protective avirulent and the non-protective avirulent—were being seeded from day to day on the various media that have already been described. After a time morphological and serological differences were noted which could only be ascribed to continuous subculturing.

It was observed that the non-protective avirulent strains 'Q' and '120/5II', grown on serum agar, were gradually acquiring a cover of the envelope substance. To begin with it was only a very thin layer but by the end of about 12 weeks it could be described as being moderate in amount so that the organisms could no longer be said to be non-enveloped. At the same time the character of the growth of these organisms had changed and they had acquired the typical viscidity of a normal plague culture. The power of producing the envelope substance had thus been re-acquired by these strains when grown on serum agar. It must, however, be stated that the recovery in this direction was not complete since at no time have these strains shown the presence of an envelope equal in degree to that possessed by the virulent strains of which they were the derivatives. The other non-enveloped strains—'I', 'P' and 'TRU'—when treated similarly remained true to their original description morphologically. The analysis of sera produced against them (*see* Part II), however, showed that they were not entirely devoid of the envelope substance and that growth on a suitable medium had resulted even in their case in an increase in the amount of this antigen although no evidence of such a change having taken place was available from the study of their morphology alone.

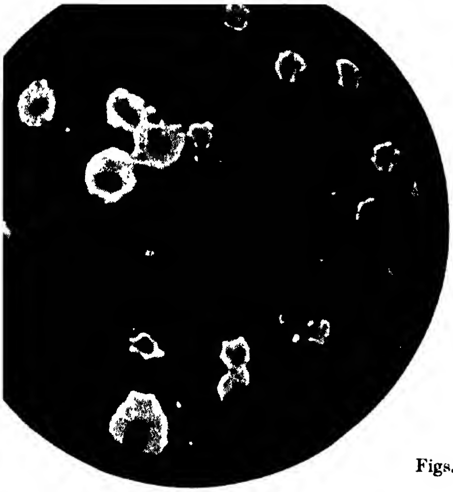
After this experience an attempt was made to degrade the enveloped strains to non-enveloped forms. A fully virulent strain—'Preanger'—and the 'rough' and 'smooth' variants of the protective avirulent strain 'Tjiwidej', all of which possessed a well-defined envelope, were subcultured on rabbit-blood agar and ordinary nutrient agar every 48 hours and incubated continuously at 37°C. After 19 subcultures strain 'Tjiwidej rough' entirely lost the property of producing envelope substance and was found to be completely bare. It has since behaved both morphologically and serologically similar to the non-enveloped strain 'TRU'.

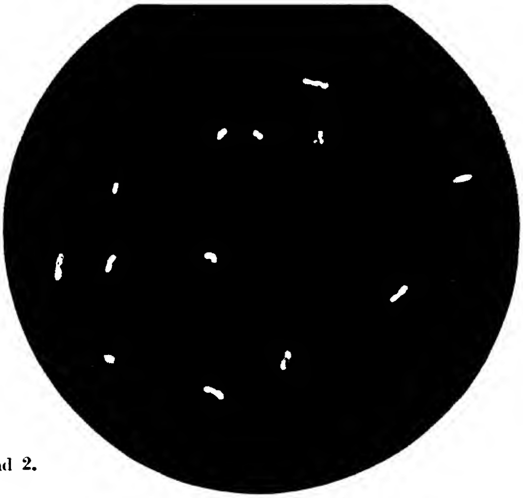
The other two strains, experimented on simultaneously, proved to be more resistant in this respect. After subculturing for over 20 weeks it was not found possible to deprive them of their envelope. It was, however, evident from a comparison with the original strains that continuous subculturing on both rabbit-blood agar and ordinary agar has a deleterious effect on the production of the envelope substance quantitatively. The most suitable medium for the optimal development of the envelope round a plague bacillus was found to be the horse serum agar the serum having been obtained from well-nourished horses.

It will thus be seen that the biological activity of the plague bacillus, so far as it relates to the production of the envelope substance, can be altered in both directions by prolonged subculturing on different media. Simultaneously with the attenuation in the virulence of the organism, maintenance on blood agar resulted in the loss of capacity to produce the envelope substance, while a similar procedure on horse-serum agar helped in the restoration of this property.

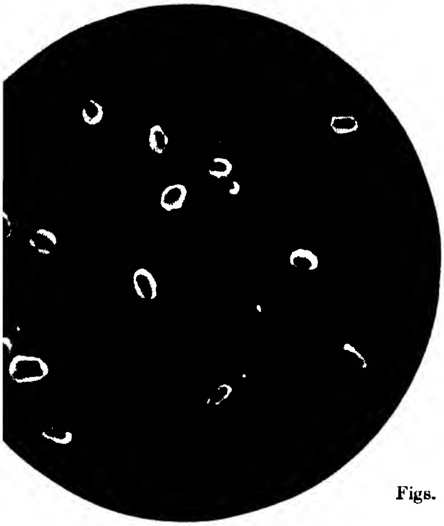
The effect of temperature of incubation and the medium of growth on the envelope and the bacterial cell.—It was consistently observed that the largest quantity of the envelope substance was produced when the temperature of incubation was 37°C. The bacterial cell developed at this temperature was fat and bipolar staining could be seen in certain strains although this property was not found to be related to virulence alone since protective avirulent strains also at times presented this appearance. The addition of horse serum and glucose to the basal media brought about an improvement both in the envelope and in the soma of the organism. The growth on glucose media, although not so luxuriant, was, however, characteristic. The bacilli were seen to be lying in bunches embedded in a common sheath of the envelope substance much thicker than could be produced on any other medium employed for this study. Increase in the period of incubation at 37°C. was accompanied by further production of the envelope substance till about the end of the first week. A simultaneous increase in the size of the bacterial body also took place. After a total of ten days' incubation, autolysis set in as a rule and after about two weeks it was difficult to identify the bacterial body inside the envelope. All that could be seen in a microscopic field at this time was a large number of empty shells of the envelope substance, a few only enclosing degenerated badly stained bacilli.

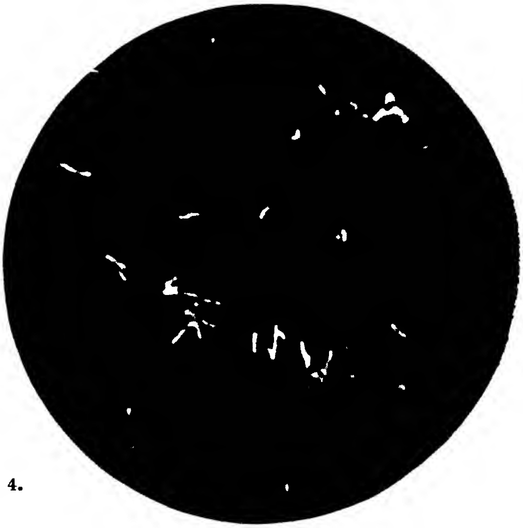
Reduction in the temperature of incubation from 37°C. was found to be accompanied simultaneously with a decrease in the amount of the envelope substance as well as in the size of the bacterial cell. When incubation at a temperature of 27°C. was employed, it was impossible to differentiate between the virulent, the protective avirulent and the non-protective avirulent groups (*see* Plate I). All the strains grew as thin rods with practically no cover of envelope substance. Prolonged incubation at this temperature, specially on media to which horse serum and glucose had been added, however, brought about a certain degree of restoration in the amount of the envelope substance and in the size of the bacterial cell, but this could in no case bear comparison with the effect of incubation at 37°C.





Figs. 1 and 2.





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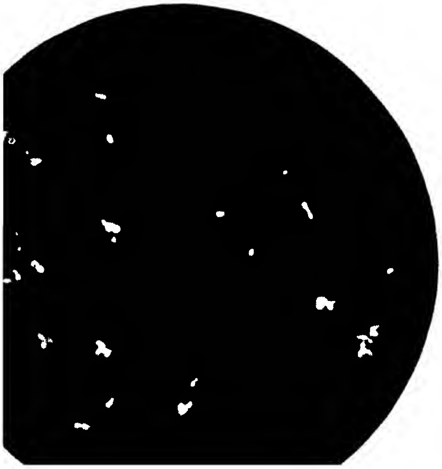

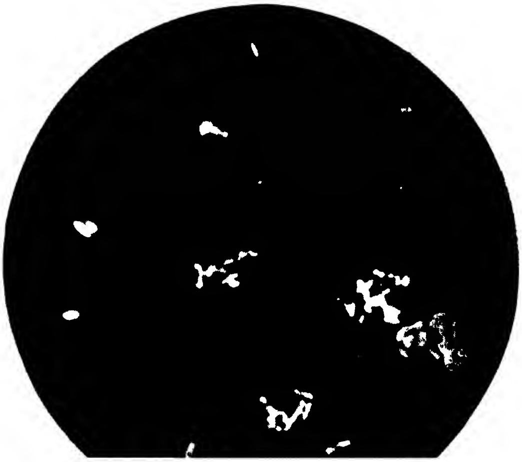

Explanation of Plate I.

(1) Fully virulent plague bacillus :—

Fig. 1.—Growth at 37°C. for 48 hours on horse-serum (10 per cent) agar.

Fig. 2.—Growth at 27°C. for 48 hours on horse-serum (10 per cent) agar.

(2) Protective avirulent plague bacillus :—

Fig. 3.—Growth at 37°C. for 48 hours on horse-serum (10 per cent) agar.

Fig. 4.—Growth at 27°C. for 48 hours on horse-serum (10 per cent) agar.

(3) Non-protective avirulent plague bacillus :—

Fig. 5.—Growth at 37°C. for 48 hours on horse-serum (10 per cent) agar.

Fig. 6.—Growth at 27°C. for 48 hours on horse-serum (10 per cent) agar.

Further reduction in the temperature of incubation intensified its effect on both the envelope substance and the bacterial body, so much so that at the temperature of the frigidaire (± 4°C.) all the strains presented a typical needle-shaped appearance, the organisms being perfectly bare. That multiplication of the bacillus takes place at the temperature of the cold room will be shown later.

No difference was noted either in the envelope or in the bacterial cell that could be said to have been brought about by the liquid or the solid nature of the substrate alone, except that the growth in broth could be readily distinguished by a characteristic chain formation of the organism.

(*b*) *The growth of* Pasteurella pestis.

A comparative series of experiments with strains representing the virulent, the protective avirulent and the non-protective avirulent groups was undertaken to determine the effect of the medium and the temperature of incubation on the total bulk of the growth of plague bacillus. Organisms were grown both in the liquid and on the solid media, with and without the addition of horse serum and glucose, and incubated continuously for four weeks at temperatures ranging from 37°C. to that of the frigidaire (± 4°C.). The difficulty of obtaining a heavy growth at higher temperatures, which is well known, was also experienced by us when this organism was grown on basal agar and broth media. Where serum had been added the growth was more luxuriant and this was considerably improved when the serum agar tubes were incubated in a position, with a slight inclination from the horizontal so that the organisms grew all the time in a moist environment of the water of condensation. The effect of glucose on the total bulk of growth was somewhat inhibitory although the increase in the envelope substance was far in excess of that produced on any other medium employed.

Reference has already been made to the fact that multiplication of the plague bacillus takes place at a temperature of ± 4°C. To exclude any error in this observation the tubes were chilled by being left in the cold room for 48 hours before they were inoculated. Although the growth was slow, more so in the case of the virulent strains, it was observed to have taken place by the end of 72 hours and was quite significant by the end of a week both on the solid and in the liquid medium. This was confirmed by the change which the organism had undergone in its morphology, this having been already described, and by its biochemical activity as shown by the fermentation of certain carbohydrates.

A comparison of the total amount of growth at different temperatures was in accordance with observations made by other workers (Sokhey, 1932-35) that the largest bulk is obtained round about a temperature of 27°C. It has already been stated that the production of the envelope substance at this temperature is comparatively poor even after prolonged incubation. In view of the dominant rôle of this structure in plague immunity (Schütze, 1932) it would appear that the optimum temperature for quantitative growth of the plague bacillus does not correspond with its qualitative optimum in terms of its immunity reactions. It is, therefore,

essential that this fact must be taken into consideration in the determination of the temperature of incubation for the preparation of a plague prophylactic.

(c) *The dissociative phenomena in* Pasteurella pestis.

The possession of cultures of varying virulence presented an opportunity to study the dissociation of the plague bacillus and to determine at the same time any differences in the colonial appearance which could be associated with this variation.

Fresh saline suspensions of representative strains of the virulent, the protective avirulent and the non-protective avirulent groups were at first prepared. A drop from each was then plated on ordinary nutrient agar, rabbit-blood agar and horse-serum agar, and incubated at 37°C. and at 27°C. The size of the drop was so adjusted that only 50 to 100 colonies developed on a plate. This procedure required preliminary experimentation and a knowledge of the intrinsic characters of individual strains since all the strains belonging to a particular group did not grow equally at the same temperature of incubation. The colonies thus obtained were further studied by replating and their characters observed both by reflected and by transmitted light and with the aid of a dissecting microscope. The following types were encountered in the strains examined :—

(1) *Dew-drop type.*—These colonies were circular, and clear cut, translucent to opaque in appearance with no halo or fringe and sticky in character. Stained Indian ink preparations showed ovoid bacilli with a good cover of the envelope substance.

They were observed to develop better at 37°C. than at 27°C. and especially on media which contained blood and serum.

The dew-drop colony was encountered mostly during the first 24 hours of growth. After this period it usually gave rise to three subtypes, namely, (*a*) a slightly larger hemispherical opaque colony with a transparent halo round it, (*b*) a large yellowish opaque colony consisting of a large central heaped up circular mass with a transparent fringe round it, and (*c*) delicate transparent white plaques of the type described by Dieudonne and Otto (1928).

These changes in the appearance of the parent colony could only be detected by frequent examination of the plates since in a good many cases the various subtypes were also seen to originate *de novo*. Subtype (*b*), i.e., an opaque colony with a fringe, was the common variant of the parent colony after 48 hours' incubation. While the other characters of subtypes (*a*) and (*b*) remained the same as that of the parent colony, their individual organisms presented a longer appearance. The white plaques of subtype (*c*), on the other hand, represented a degenerative change in the organism since the bacilli were thinner and longer and almost devoid of the envelope substance.

(2) '*Smooth*' *type.*—This colony was larger and flatter than the dew-drop type, transparent to translucent in appearance and usually circular although some ovoid forms were also seen. A few were seen to possess a raised centre. This type was

not so sticky as the dew-drop type. Stained Indian ink preparations showed cylindrical bacilli with an average amount of the envelope substance. The organisms stained better than those of the dew-drop type.

The growth was more luxuriant at 27°C. than at 37°C. The colonies could be easily identified on ordinary nutrient agar where they resembled the smooth type of growth of the typhoid-paratyphoid group of organisms. The growth on serum agar and on blood agar was either typical. or confluent or tiny colonies.

Two subtypes of this colony were observed, namely, (*a*) a colony, which, though it resembled the parent colony in appearance, had a gritty feeling when touched with a platinum loop and its suspension in normal saline solution, or even in water, was unstable. Indian ink preparations showed thin and long bacilli with practically no cover of the envelope substance; (*b*) delicate, transparent white plaques of the type described by Bezsonowa and Lenskaja (1930-31). Their only distinction from subtype (*c*) of the dew-drop colony was that they were observed to develop from the 'smooth' type of colony, otherwise they presented a similar degenerative change, the organisms being long, thin and perfectly bare.

(3) *Other types of colonies.*—A variety of colonies presenting definite naked-eye and morphological characters are grouped under this heading. They could not be considered to be true mutations of the plague bacillus since from the point of view of bacterial cyclogeny they did not possess any hereditary constancy about them. Besides, a number of them did not represent a single morphological type of bacillus but were mixtures of at least two different types of organisms. This applied particularly to colonies which resembled the 'sunflower form' described by Bezsonova, Somekoz and Kotelnokov (1927). The following is a description of some of the types observed :—

(*a*) *Opaque colony.*—Three different varieties of this colony were encountered, namely,

(*i*) opaque colony with raised centre,
(*ii*) opaque circular colony, and
(*iii*) dense opaque colony.

(*i*) The type with raised centre looked perfectly transparent when first isolated but on replating assumed a yellowish opaque hue. The growth on nutrient agar was luxuriant and presented a moist appearance; but on trying to pick the colony it was found to be perfectly dry and difficult to emulsify. Indian ink preparations showed fat organisms, like *pneumococci*, some of them being in chains but all of them devoid of any envelope substance.

(*ii*) The opaque circular colony was also dry in character and difficult to emulsify. It showed luxuriant growth at 37°C. after 48 hours on nutrient agar with a certain amount of yellow chromogenesis. On replating, in addition to the parent type, it gave rise to transparent and translucent white colonies. While the organisms of the parent colony were fat, mostly in clumps showing good bipolar staining, those of the variants were long and thin. All of them were almost devoid of the envelope substance.

(*iii*) The dense white colony gave a yellowish white growth on nutrient agar which was more luxuriant when the temperature of incubation was 27°C., rather than 37°C. It was not so difficult to emulsify. The growth in broth was accompanied with pellicle formation. Indian ink preparations showed long thin rods representing typical involution forms which were perfectly bare.

(*b*) *Bluish transparent colony.*—These were tiny colonies which were difficult to pick up. They gave rise to very sparse growth both at 37°C. and at 27°C. in all the media. The best growth was obtained in broth at 27°C. after 48 hours the medium being uniformly turbid. Indian ink preparations showed tiny ovoid organisms with no cover of the envelope substance.

(*c*) *Sunflower type.*—The typical colonies of this type consisted of yellowish white more or less circular heaped up inner mass and a thick fringe-like periphery with radial ridging. They developed best on blood media at 37°C. Indian ink preparations showed two types of bacilli: (*a*) typical fat ovoid organisms with a good cover of the envelope substance, and (*b*) long, thin rods with practically no cover. On replating, in addition to the parent colony, they gave rise to dew-drop type, 'smooth' type and various other types of colonies.

All the varieties of colonies described here were seen to be produced from *all the strains* of *Pasteurella pestis* some time or other during the course of this study. Since their naked-eye characters and the morphological appearance of the bacilli contained in them, both from the point of view of the bacterial cell and that of the envelope substance, have been shown to differ, the plague bacillus affords an excellent example in support of the contention of Hadley (1927) that the bacterial population of a culture is not made up of organisms which share equally all the characters of the species to which the culture belongs. In other words, microbic dissociation is typically exemplified by the cultural behaviour of *Pasteurella pestis* as judged by the study of colonies produced by it.

Although it was not found possible to correlate exactly the virulence or the avirulence of a particular *Pasteurella pestis* strain and the type or the types of colonies produced by it, it was, however, noticed that quantitatively such an association did exist. The virulent strains were observed to produce the largest number of dew-drop type of colony and specially its variant the opaque colony with a transparent fringe. The avirulent strains, on the other hand, were characterized by a preponderance of the 'smooth' circular type of colony. Even differentiation between the protective and the non-protective avirulent strains could be made out by a study of the 'smooth' type of colony. The non-protective avirulents were observed to produce always a larger number of that variant of this colony which has already been described as giving a gritty feeling when touched with a platinum loop and which, when emulsified, gave rise to an unstable suspension.

Attempts at isolating pure forms of the dew-drop and the 'smooth' type of colony failed consistently since a single colony of either type always produced an admixture of the two types besides other varieties of the colonies. It was impossible to separate any true breeding forms from other types of colonies.

Summary.

1. A large number of strains of *Pasteurella pestis* were collected from different parts of the world with a view to a systematic investigation of this organism. Part I deals with the study of their morphology, their growth on different media and at different temperatures of incubation, and their colonial appearance. These strains could be divided into a virulent group and an avirulent group. The latter group was further classified into: (*a*) those strains whose live suspensions have been employed in mass human inoculation for protection against plague; these were termed by us as ' protective avirulent strains ' and (*b*) those strains which did not give rise to any such protection. To these the term ' non-protective avirulent strains ' has been applied.

2. The plague bacillus does not possess a capsule of the same nature as that of other organisms like the *pneumococcus*. Its morphology, on the other hand, studied by the methods described, brings out two separate structures, namely, (*a*) the bacterial body itself and (*b*) a halo round it which is more of the nature of an envelope than a capsule.

3. Both the virulent and the protective avirulent strains were possessed of a well-defined envelope. They could be distinguished morphologically only by quantitative differences in the amount of their envelope substance. In contrast to them, the non-protective avirulent strains did not show the presence of an envelope irrespective of the medium of growth and the temperature of incubation.

4. The most suitable medium for the optimal development of the plague bacillus, both from the point of view of the envelope and the bacterial body, was found to be 10 per cent horse-serum agar. The addition of a carbohydrate to this medium resulted in the production of a large amount of the envelope substance but the total growth was not so luxuriant. Continued subculturing on rabbit-blood agar led eventually to the loss of the envelope cover. The liquid or the solid nature of the substrate did not make any difference to the production of the envelope substance or the size of the bacterial cell. A characteristic chain formation of the organisms, however, took place when the medium was liquid.

5. Incubation at a temperature of 37°C. brought about the optimal development of both the envelope and the bacterial body. Reduction in this temperature was accompanied by a simultaneous decrease in the amount of the envelope substance as well as the bacterial cell. When the temperature of incubation was 27°C. it was impossible to differentiate between the organisms belonging to the virulent, the protective avirulent and the non-protective avirulent groups although the total bulk of growth in the case of all the strains was much more at this temperature than at any other. At the temperature of the cold room ($\pm$ 4°C.) multiplication of the organisms took place but all the strains grew as typical needle-shaped bacilli completely devoid of the envelope substance.

6. A large variety of colonial types have been described to which all the strains examined gave rise at some time or other in the course of this study. Although it was not possible to correlate the virulence or the avirulence of a strain with a

particular type or types of colonies, an indication in this direction was nevertheless obtained from certain definite types some of which preponderated in a virulent and others in an avirulent culture.

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BACTERIOLOGICAL STUDIES ON *PASTEURELLA PESTIS* AND *PASTEURELLA PSEUDOTUBERCULOSIS*.

Part II.

THE SEROLOGY OF *PASTEURELLA PESTIS* AND *PASTEURELLA PSEUDOTUBERCULOSIS*.

BY

MAJOR S. S. BHATNAGAR, M.D., Ph.D., F.R.C.P., I.M.S.

(*From the Central Research Institute, Kasauli.*)

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THE plague and the pseudotuberculosis antigens and their serum counterparts have been studied by the methods of agglutination and of agglutinin absorption. Agglutination of the plague bacillus has always been accompanied by difficulties mainly in regard to: (*a*) the preparation of stable suspensions and (*b*) the production of immune sera of high agglutinating titres. Different methods have been suggested by different workers (Wherry, 1905; Strong and Teague, 1912; Signorelli and Caldarola, 1912; Kling and Hesser, 1921; Pulgher, 1922; D'Aunoy, 1923; Batchelder, 1929; Pirie, 1929; de Smidt, 1929-30; Greval and Dalal, 1933; Brigham and Rettger, 1935; Wats, Wagle and Puduval, 1939; and others). The plague bacillus was subjected by them to such diverse procedures as the extraction of its antigens, its growth at lower temperatures and its agglutination in low salt concentrations. From a consideration of the physico-chemical factors involved in the reaction of agglutination, it appeared that reliable results could only be obtained by the practice of a technique in which the spatial configuration of the antigens of this organism was disturbed as little as possible and its agglutination carried out under conditions which corresponded with those employed in similar studies on other organisms. With these objects in view a technique of agglutination was developed which has given consistently reproducible results.

(*a*) *A new method for agglutination tests of* Pasteurella pestis *and allied organisms.*

The principles involved in the practice of this technique may be briefly stated to be: (1) the organism is grown under conditions which lead to the optimum development of the envelope substance and the optimum growth of the bacillus

itself, (2) multiple strains representing different characters are employed with the dual object of obtaining specific agglutination as well as the maximum degree of agglutinability, (3) the agglutinating suspension is used in a fresh and a live state and is so prepared that there is the least likelihood of any physical or chemical alteration in the nature of the antigen or antigens and (4) the agglutination is carried out in physiological normal saline solution without the necessity of reducing salt concentration.

(*i*) *The medium of growth and the temperature of incubation.*—The plague bacillus is grown on 10 per cent horse-serum agar for 48 hours at 37°C. The tubes are incubated in an almost horizontal position with a slight inclination so that the whole agar surface is kept moist by the water of condensation. The seeding of the organism on to slopes which do not possess sufficient moisture must be avoided.

(*ii*) *The selection of strains for the test.*—As a result of the study of the morphology of the plague bacillus the strains of this organism were divided into enveloped and non-enveloped forms. With their help it was considered that it would be possible to obtain agglutination both of the envelope substance and that of the soma of the bacillus.

With the enveloped strains a great variability in the degree of their agglutinability was observed, the difference being as much as five times. On this basis all the strains could be classified into three groups, namely, (*a*) those showing a minimum degree of agglutinability, (*b*) those which could be said to occupy an intermediate position in this respect, and (*c*) those which possessed the maximum sensitivity in this type of reaction. This classification corresponded more or less closely with the division of these strains into virulent, protective avirulent and the non-protective avirulent groups. The only members of the last group which were stable in normal saline solution were the strains 'Q' and '120/5H' which have been shown to have reacquired the property of producing envelope substance after prolonged subculturing on serum agar.

One strain from each group was selected for the study of envelope agglutination, namely, 'Preanger', 'Tjiwidej smooth' and 'Q'. These were employed throughout the course of this study.

Neither the enveloped nor the non-enveloped strains could, however, be utilized for the study of somatic agglutination of plague bacillus. In enveloped forms the presence of the envelope substance on the surface of the organism interfered completely with this reaction taking place. Where this structure was absent, although somatic agglutination took place the results could not be interpreted correctly because of the salt and serum sensitivity of such suspensions even in as low a salt concentration as 0·1 per cent. In view of this experience it may be assumed that it is the envelope substance which bestows salt stability on a suspension of the plague bacillus.

A chance observation arising out of cross-agglutination tests on *Pasteurella pestis* and *Pasteurella pseudotuberculosis* strains suggested the possibility that the latter type of organism may prove helpful in estimating the somatic titre of anti-plague sera. Experiments were repeated and they demonstrated conclusively

that such was the case. The serological relationship between these two organisms will be discussed later. Comparative tests on the sensitivity of various types of *Pasteurella pseudotuberculosis* strains revealed that strains belonging to group I, types A and B (Schütze, 1928) gave the highest readings in agglutination tests against anti-plague sera. One strain each belonging to these two types was selected and employed throughout.

(*iii*) *The preparation of agglutinating suspension.*—About 5 c.c. of sterile normal saline solution was poured on to each serum-agar slope and the growth gently emulsified into it with the help of a platinum loop. It was then rotated between the palms of both hands for a few seconds and allowed to stand at room temperature for two hours. During this time that part of the growth which had not gone into suspension deposited at the bottom of the tube. The supernatant fluid was then pipetted off and its density adjusted to that of Brown's opacity tube No. 2 in the case of the plague bacillus and to that of No. 3 for the organism of pseudotuberculosis. Such suspensions were found to be perfectly stable, no sediment being deposited after 7 days when left on the bench. Looked at under dark-ground illumination they were seen to be made up of single organisms. The live suspensions were used on the same day as prepared.

(*iv*) *The agglutination test.*—Graduated dilutions of the serum were set up in a series of tubes, about 5 cm. long with a round bottom and with a uniform internal diameter of 1·25 cm. of the type used by Felix for typhoid group agglutination. The usual order of serum dilutions was 1 : 10, 1 : 25, 1 : 50, 1 : 100, 1 : 200, 1 : 500 and so on. The total volume of the serum-suspension mixture was 1·0 c.c. in each case. This was made up by adding to the serum the requisite number of drops of the suspension with the help of a dropping pipette which was so graduated that 20 drops equalled 1·0 c.c.

The tubes were left in the 37°C. incubator overnight and then at room temperature, the total period being 24 hours. From a large number of experiments at different temperatures and for different periods of time, it was observed that the highest degree of flocculation was given by this procedure.

(*v*) *Readings.*—The various degrees of agglutinability were expressed as follows :—

+++ Strongest degree of agglutination; supernatant fluid completely clear.

++±, ++, +±, + Degrees of incomplete agglutination; supernatant fluid turbid: the strength of the reaction is judged according to the amount of sediment visible to the naked eye.

± Weakest degree of agglutination which can be estimated by the naked eye.

(±), [(±)] Trace and faint trace readings estimated by means of a magnifying lens (10 ×).

Control.—Complete absence of flocculation; a deposit of non-agglutinated organisms takes place giving a compact mass in the centre of the bottom of the control tube similar to the control tubes in the case of Salmonella agglutination. This should not be taken to be as evidence of salt instability of the organism.

In agglutination of the plague bacillus and that of the organism of pseudotuberculosis it is unusual to obtain the serial readings from +++ (three plus)

to [(±)] (faint trace) as is the case in a similar test with the Salmonella group of organisms. With envelope agglutination it was our common experience to obtain a +++ (three plus) reading followed by a + (one plus) or [(±)] (faint trace) or even complete absence of agglutination in the next tube. The determination of the end point, therefore, presented some difficulty and different strains of *Pasteurella pestis* with different degrees of sensitivity for this type of reaction had to be employed. The standard envelope titre was taken to be that dilution of the serum which gave a +++ (three plus) reading against strain 'Q' belonging to the non-protective avirulent group of the plague bacillus, a strain which has already been described as giving rise to the highest degree of envelope agglutinability. The weaker degrees of flocculation were, however, also taken into consideration especially when judging the relative potency of two different sera for animal experiments to be described later. In the case of agglutination of *Pasteurella pseudotuberculosis* strains the drop in the amount of sediment with serial dilutions of the serum was more gradual. The standard somatic titre was taken to be that dilution of serum which gave a + (one plus) reading. It is necessary to emphasize that continuous subculturing results in the diminution of that antigenic component of *Pasteurella pseudotuberculosis* which is common to it and the plague bacillus. It is, therefore, essential that a strain which has been subcultured for more than approximately 6 weeks should be replaced by the original parent strain at the end of this period in order to obtain the best results from the reaction of agglutination.

The characteristics of the two types of agglutination may be tabulated as follows :—

Envelope agglutination.	Somatic agglutination.
(1) Forms slowly.	(1) Forms slowly.
(2) Settles slowly.	(2) Settles slowly.
(3) Supernatant clear.	(3) Supernatant clear.
(4) Sediment voluminous, flakes large, woolly in character and varying in size.	(4) Sediment scanty, flakes small, uniform and gritty in character.
(5) In lower dilutions sediment easily dislodged and produces a shimmer in the serum-suspension mixture; in higher dilutions definite woolly particles visible.	(5) Sediment easily dislodged and becomes similar to saline control on shaking in all the dilutions of serum.

The characters of the two types of agglutination can be studied more satisfactorily if formalin (0·1 per cent) is added to the normal saline used for the preparation of agglutinating suspensions. Under the effect of this chemical the sediment of agglutinated bacilli both with *Pasteurella pestis* and *Pasteurella pseudotuberculosis* does not go back into suspension on shaking, as is the case with live organisms, but retains the appearance of individual particles. In view, however, of the deleterious effect of formalin on the degree of agglutinability (*see* Table VII) this reagent cannot be recommended to form part of the serum-suspension mixture.

(*b*) *The production of high-titre sera against* Pasteurella pestis *and* Pasteurella pseudotuberculosis.

Just as with the agglutination tests so also with the immunization of rabbits it was desired that the sera should be raised against antigens in which the possibility

of physical or chemical alteration was reduced to a minimum. With this object in view live bacteria belonging to the protective avirulent and the non-protective avirulent groups were employed. Sera against the virulent group of *Pasteurella pestis* and the various types of *Pasteurella pseudotuberculosis* could not, however, be produced in this manner because of the risk of losing animals. A method was, therefore, looked for which precluded the use of heat and disinfectants, such as carbolic acid, in view of the fact that immunizing suspensions so treated did not give rise to satisfactory envelope and somatic titres. Various other sterilizing reagents were, therefore, tried. The one which was found to be most satisfactory was silver nitrate as employed by Rainsford (1939) for T.A.B. vaccine. Briefly described the method is as follows :—

The serum-agar growth of the organism was suspended in distilled water and its opacity so adjusted that 8 to $10{,}000 \times 10^6$ bacilli were contained in 1 c.c. A desired amount of this suspension was mixed with an equal volume of 0·004 per cent aqueous solution of silver nitrate. The mixture was incubated overnight at 37°C. Next morning sufficient NaCl solution (10 per cent) was added to render the suspension isotonic. Having been well shaken the suspension was left in the frigidaire. Sterility tests were commenced after 24 hours. Such suspensions were usually sterile after 72 hours. The final concentration of the organisms in such a suspension was 4 to $5{,}000 \times 10^6$ per c.c. and that of silver nitrate 0·002 per cent.

Attention must be drawn to the danger of serum anaphylaxis where organisms grown on serum media are injected into a rabbit. This phenomenon was found to start a few minutes after the injection. The animal became restless, the tone of the muscles increased quickly and death accompanied by respiratory dyspnœa ensued. Where the animal lived for a few hours loss of power in the limbs was observed. To overcome this difficulty all the animals before the commencement of immunization were de-sensitized with 0·25 to 0·4 c.c. of horse serum intramuscularly. With this procedure no animals were lost.

Dosage.—It was found necessary to give a prolonged and repeated course of injections to obtain a high-titre serum. An initial high dose was well tolerated by the animals and it was observed that toxicity did not bear any relationship to the virulence of the strain used. In fact it was the experience that the non-enveloped forms were more toxic than the virulent strains. While higher doses of the latter type of organism could be injected without any ill effects such was not the case with the other type. No negative phase was noticed, a recognizable somatic titre being present as early as 48 hours after the injection. The envelope agglutinins, on the other hand, took longer to develop. All the injections were given intravenously.

(*a*) *Virulent group.*—The first course consisted of 9 injections of silver-killed plague bacilli suspension arranged as follows :—

3 injections	$5{,}000 \times 10^6$	bacilli	Interval between injections 48 hours.
3 ,,	$10{,}000 \times 10^6$	,,	
3 ,,	$15{,}000 \times 10^6$	,,	

The animal was bled on the sixth day after the last dose and a titre of 1/200 to 1/500 envelope and 1/1,000 to 1/2,000 somatic agglutinins had developed by then. With intervals of about a fortnight a second and a third course was given. It was,

however, not found possible to increase the envelope titre beyond 1/1,000 although a titre of 1/50,000 somatic agglutinins could be produced by multiple courses.

(*b*) *Protective and non-protective avirulent groups.*—These were injected as live suspensions of a 48-hour growth on serum agar in the following manner :—

¼ slope	.. .. ..	3 injections.
½ ,,	.. .. ..	3 ,,
1 ,,	.. .. ..	3 ,,

While the interval between injections of ¼ and ½ slope was two days it had to be increased to from 5 to 7 days when a full slope growth was given as the animals became sick and lost weight. It was, however, essential to follow this procedure to obtain high-titre sera. After a rest lasting for about a fortnight it was possible to start a second course. As much as the total growth of 20 agar slopes has been injected into a rabbit weighing 2,000 grammes.

(*c*) *Pasteurella pseudotuberculosis.*—The procedure followed here was the same as in the case of the virulent strains of *Pasteurella pestis.* While the animals stood well the immunization with strains belonging to groups I and II and their subtypes, group III human strain was found to be very toxic for the rabbit and several animals were lost before a satisfactory serum could be obtained.

(*c*) *The antigen-antibody reactions of* Pasteurella pestis *and* Pasteurella pseudotuberculosis.

For the purpose of this study in addition to the sera prepared in the manner already described, the following sera obtained from other sources were also employed :—

(1) Bombay horse sera from animals immunized with (*a*) supernatant fluid of Haffkine vaccine, (*b*) live avirulent strains, and (*c*) live virulent strains (supplied by Lieut.-Colonel S. S. Sokhey, I.M.S., Director, Haffkine Institute, Bombay).

(2) Concentrated anti-plague Lister serum prepared in horses by immunizing them against Otten's protective avirulent 'smooth' strains injected both as killed and as live suspension (supplied by Dr. G. F. Petrie, Bacteriologist-in-Charge, Lister Institute, Elstree, England).

(3) Human convalescent plague sera (supplied by Captain C. L. Ahluwalia, Special Plague Officer, Hyderabad-Deccan).

Cross agglutination and agglutinin absorption tests between Pasteurella pestis *and* Pasteurella pseudotuberculosis.

Different types of *Pasteurella pestis* and various groups of *Pasteurella pseudotuberculosis* strains were tested for agglutination against (*a*) anti-plague sera of different origin produced in horses and in rabbits against the live and the killed plague bacilli and a human convalescent serum from a case of plague, and (*b*) four pseudotuberculosis sera prepared by immunizing rabbits with strains belonging to groups I and II of this organism. The results are shown in Tables I and II :—

TABLE I.

Agglutination of Pasteurella pestis *and* Pasteurella pseudotuberculosis *strains by anti-plague sera.*

Anti-plague serum.	Dilution.	*PASTEURELLA PESTIS* STRAINS.			*PASTEURELLA PSEUDOTUBERCULOSIS* STRAINS.				
		VIRULENT.	PROTECTIVE AVIRULENT.	NON-PROTECTIVE AVIRULENT.	GROUP I.		GROUP II.		GROUP III.
		Preanger.	Tjiwidej 'smooth'.	'Q'.	IA.	IB.	IIA.	IIB.	
Bombay horse No. 16.	25	+++	+++	+++	+++	+++	++	++	++
	50	+++	+++	+++	+++	+++	++	+	+±
	100	+++	+++	+++	+++	++±	+±	−	+
	200	+++	+++	+++	++	+	+	−	−
	500	(±)	+++	+++	−	−	−	−	−
	1,000	−	−	+++	−	−	−	−	−
	2,000	−	−	−	−	−	−	−	−
Bombay horse No. 13.	25	+++	+++	+++	+++	+++	++	−	++
	50	+++	+++	+++	++	+	++	−	+
	100	+++	+++	+++	+	−	+	−	−
	200	(±)	+++	+++	−	−	−	−	−
	500	−	−	+++	−	−	−	−	−
	1,000	−	−	−	−	−	−	−	−
	2,000	−	−	−	−	−	−	−	−
CONTROL ..		−	−	−	−	−	−	−	−

TABLE I—*concld.*

Anti-plague serum.	Dilution.	*PASTEURELLA PESTIS* STRAINS.			*PASTEURELLA PSEUDOTUBERCULOSIS* STRAINS.				
		VIRULENT.	PROTECTIVE AVIRULENT.	NON-PROTECTIVE AVIRULENT.	GROUP I.		GROUP II.		GROUP III.
		Preanger.	Tjiwidej 'smooth'.	'Q'.	IA.	IB.	IIA.	IIB.	
Lister ..	25	+++	+++	+++	+++	+++	+++	++	+++
	50	+++	+++	+++	+++	+++	++	++	+
	100	+++	+++	+++	+++	++±	+	+	–
	200	–	+++	+++	+++	++	+	–	–
	500	–	–	+++	+	+	–	–	–
	1,000	–	–	–	–	–	–	–	–
	2,000	–	–	–	–	–	–	–	–
E. V.'	25	+++	+++	+++	+++	+++	+++	+++	+++
	50	+++	+++	+++	+++	+++	+++	++	+
	100	+++	+++	+++	+++	+++	++	+	–
	200	+++	+++	+++	+++	++±	+	–	–
	500	–	+++	+++	+++	++	+	–	–
	1,000	–	–	+++	+++	+	–	–	–
	2,000	–	–	–	++	–	–	–	–

'I' ..	25	+	+−	+++	+++	+++	+++	++	+++
	50	−	+	++	+++	+++	++	++	++
	100	−	−	+	+++	+++	++	+	++
	200	−	−	−	+++	++	+	−	+
	500	−	−	−	+++	++	−	−	−
	1,000	−	−	−	++	+	−	−	−
	2,000	−	−	−	−	−	−	−	−
Human (6) ..	25	+++	+−+	+++	+++	+++	+++	+++	++
	50	++−	+−+	+−+	+++	++	+	+	++
	100	−	−	+++	+++	++	−	−	+
	200	−	−	−	++	+	−	−	−
	500	−	−	−	−	−	−	−	−
	1,000	−	−	−	−	−	−	−	−
	2,000	−	−	−	−	−	−	−	−
Control ..		−	−	−	−	−	−	−	−

TABLE II.

Agglutination of Pasteurella pestis *and* Pasteurella pseudotuberculosis *strains by pseudotuberculosis sera.*

Pseudo-tuberculosis serum.	Dilution.	*PASTEURELLA PESTIS* STRAINS.						*PASTEURELLA PSEUDOTUBERCULOSIS* STRAINS.				
		VIRULENT.				PROTECTIVE AVIRULENT.	NON-PROTECTIVE AVIRULENT.	GROUP I.		GROUP II.		GROUP III.
		Java.	South Africa.	Japan.	Bombay.	Tjiwidej 'smooth'.	Bombay 'Q'.	IA.	IB.	IIA.	IIB.	
Group IA	10	−	−	−	−	−	−	++±	+++	+++	+++	+++
	25	−	−	−	−	−	−	+++	+++	+++	++±	++
	50	−	−	−	−	−	−	+++	+++	++	++	++
	100	−	−	−	−	−	−	+++	+++	++	+	++
	200	−	−	−	−	−	−	+++	+++	+	−	+
	500	−	−	−	−	−	−	+++	++±	−	−	−
	1,000	−	−	−	−	−	−	+++	++	−	−	−
Group III	10	−	−	−	−	−	−	+++	+++	+++	+++	+++
	25	−	−	−	−	−	−	+++	+++	++	++	+++
	50	−	−	−	−	−	−	++	++	++	++	+++
	100	−	−	−	−	−	−	++	+	+	+	++
	200	−	−	−	−	−	−	+	+	−	−	−
	500	−	−	−	−	−	−	−	−	−	−	−
	1,000	−	−	−	−	−	−	−	−	−	−	−
CONTROL ..		−	−	−	−	−	−	−	−	−	−	−

Tables I and II demonstrate a divergence in the behaviour of the two organisms towards the two types of sera. Whereas the anti-plague sera flocculate both the plague bacillus and the organism of pseudotuberculosis, none of the strains of *Pasteurella pestis* react with the pseudotuberculosis sera. This was found to be the case with all the plague strains tested against sera of all the groups of *Pasteurella pseudotuberculosis.* By a study of its morphology it was noted that the organism of pseudotuberculosis is devoid of the envelope substance which ensheaths the surface of a normal plague bacillus. It may, therefore, be concluded that the agglutinability of plague bacillus by an anti-plague serum represents an antigen-antibody reaction between the surface envelope antigen of this organism and the corresponding antibody in the serum. A similar reaction between an anti-plague serum and the organism of pseudotuberculosis must be due to an entirely different antibody in the serum whose antigen counterpart is also present in *Pasteurella pseudotuberculosis.* In other words, the two organisms are possessed of a common antigen.

Following the terminology introduced by Schütze (1932) the reaction between the plague bacillus and its homologous serum may be designated as envelope antigen-antibody reaction and that between an anti-plague serum and *Pasteurella pseudotuberculosis* as somatic antigen-antibody reaction.

The non-agglutinability of the plague bacillus by a pseudotuberculosis serum may be accounted for by the absence of envelope antibody from the serum. As has already been stated, it is not possible to demonstrate a somatic type of agglutination between the plague bacillus and either an anti-plague or an anti-pseudotuberculosis serum since the presence of the envelope antigen on the surface of the organism precludes the possibility of such an interaction. Where this structure was noticed morphologically not to be present, such as in certain members of the non-protective avirulent group, its absence made the suspension of such plague bacilli salt sensitive in as low a concentration as 0·1 per cent. Consequently, it was not possible to interpret correctly the results of such somatic plague antigen-antibody reactions.

It will be seen from Table I that animal immunization with *Pasteurella pestis* strains results in the production of a considerable amount of both the envelope and the somatic agglutinins which can be differentiated by the new method of agglutination in which both *Pasteurella pestis* and *Pasteurella pseudotuberculosis* strains are employed. The serum prepared against strain ' I ', a member of the non-protective avirulent group, gave a rich somatic and a poor envelope titre. Morphological observations have already shown that this group is devoid of the envelope substance. The production of a small envelope titre on animal immunization, which was the experience with all the members of this group including the strain ' TRU ', would indicate that a small fraction of the envelope antigen, not detectable by a study of their morphology, is, however, possessed by such organisms.

The examination of a number of sera of human cases recovering from plague also showed that both the envelope and the somatic agglutinins were present in them although their titre was commonly found to be low.

Attention must be drawn to a considerable drop in the titre of both types of agglutinins, somatic as well as envelope, when anti-plague sera were stored for a length of time. Although a certain degree of deterioration under such conditions usually takes place, in the case of these sera it was observed to be out of proportion to the period for which they were stored in the cold room; this was especially so where trikresol or phenol had been added as a preservative. In view of this observation the effect of storage on the potency of anti-plague sera, particularly those placed on the market for therapeutic purposes, needs special investigation.

In order to elucidate further the serological relationship between *Pasteurella pestis* and *Pasteurella pseudotuberculosis*, cross agglutinin absorption tests were carried out. Sera were absorbed reciprocally and their agglutinability tested after absorption. The results obtained in one of these experiments, where an anti-plague serum was absorbed by the various groups of *Pasteurella pseudotuberculosis*, are shown in Table III. Absorption of pseudotuberculosis sera by the plague bacillus is discussed later (*see* Table V) since it raises issues which relate to the serological inter-relationships between the various groups of *Pasteurella pseudotuberculosis.*

TABLE III.

Agglutination by an anti-plague serum after its absorption by various sub-groups of Pasteurella pseudotuberculosis.

Anti-plague serum Preanger'.	Dilution.	*PASTEURELLA PESTIS* STRAINS.			*PASTEURELLA PSEUDO-TUBERCULOSIS* STRAINS.				
		Preanger.	Tjiwidej 'smooth'.	'Q'.	GROUP I.		GROUP II.		GROUP III.
					IA.	IB.	IIA.	IIB.	
Unabsorbed	25	+++	+++	+++	+++	+++	+++	+++	+++
	50	+++	+++	+++	+++	+++	++±	++	++
	100	+++	+++	+++	+++	++±	+	–	+
	200	(±)	+++	+++	++	++	–	–	–
	500	–	–	+++	++	+	–	–	–
	1,000	–	–	–	+	–	–	–	–
CONTROL ..		–	–	–	–	–	–	–	–

TABLE III—*contd.*

Anti-plague serum 'Preanger'.	Dilution.	*PASTEURELLA PESTIS* STRAINS.			*PASTEURELLA PSEUDO-TUBERCULOSIS* STRAINS.				Group III.
					Group I.		Group II.		
		Preanger.	Tjiwidej 'smooth'.	'Q'.	IA.	IB.	IIA.	IIB.	
Absorbed with IA.	25	+++	+++	+++	—	—	—	—	—
	50	+++	+++	+++	—	—	—	—	—
	100	+++	+++	+++	—	—	—	—	—
	200	—	++	+++	—	—	—	—	—
	500	—	—	++	—	—	—	—	—
	1,000	—	—	—	—	—	—	—	—
Absorbed with IB.	25	+++	+++	+++	—	—	—	—	—
	50	+++	+++	+++	—	—	—	—	—
	100	+++	+++	+++	—	—	—	—	—
	200	—	++	+++	—	—	—	—	—
	500	—	—	+++	—	—	—	—	—
	1,000	—	—	—	—	—	—	—	—
Absorbed with IIA.	25	+++	+++	+++	—	—	—	—	—
	50	+++	+++	+++	—	—	—	—	—
	100	+++	+++	+++	—	—	—	—	—
	200	—	++	+++	—	—	—	—	—
	500	—	—	++	—	—	—	—	—
	1,000	—	—	—	—	—	—	—	—
CONTROL ..		—	—	—	—	—	—	—	—

TABLE III—*concld.*

Anti-plague serum 'Preanger'.	Dilution.	*PASTEURELLA PESTIS* STRAINS.			*PASTEURELLA PSEUDO-TUBERCULOSIS* STRAINS.				GROUP III.
					GROUP I.		GROUP II.		
		Preanger.	Tjiwidej 'smooth'.	'Q'.	IA.	IB.	IIA.	IIB.	
Absorbed with IIB.	25	+++	+++	+++	–	–	–	–	–
	50	+++	+++	+++	–	–	–	–	–
	100	+++	+++	+++	–	–	–	–	–
	200	–	++	+++	–	–	–	–	–
	500	–	–	++	–	–	–		–
	1,000	–	–	–	–	–	–	–	–
Absorbed with III.	25	+++	+++	+++	–		–	–	–
	50	+++	+++	+++	–	–	–	–	–
	100	+++	+++	+++	–	–	–	–	–
	200	–	+++	+++	–	–	–	–	–
	500	–	–	++	–	–	–	–	–
	1,000	–	–	–	–	–	–	–	–
CONTROL	..	–	–	–	–	–	–	–	–

It will be seen from Table III that all the strains of *Pasteurella pseudotuberculosis* completely removed the somatic agglutinin from the anti-plague serum. It would, therefore, appear that the antigen which is common to this organism and the plague bacillus is possessed by all its groups. On the other hand, the envelope agglutinin was left completely intact since the reaction of the serum to *Pasteurella pestis* strains was unaltered by such absorption.

Pure envelope and pure somatic sera.—For the purpose of determining the rôle of these two different antibodies in plague immunity it is necessary to experiment with serologically specific sera. Table III indicates how a pure envelope serum can be obtained by absorption of an anti-plague serum with any one of the strains of *Pasteurella pseudotuberculosis.* The various groups of this organism, however, do not produce equally good results, and an absorption experiment with some of them may be a very laborious process. The best results in this connection were obtained with organisms belonging to group I of this species.

The preparation of a pure somatic anti-plague serum presented considerable difficulty. Those strains of *Pasteurella pestis* which appeared to be completely bare on morphological examination, such as members of the non-protective avirulent group, gave rise on animal immunization to sera with high somatic titres but nevertheless with an envelope component, the titre of which varied from 1 in 25 to 1 in 100. The removal of this small amount of envelope agglutinins led at the same time to a considerable reduction in the somatic titre of the serum. Various methods were tried to obtain pure somatic serum without the necessity of absorption. Growth of the organism at 27°C. and at lower temperatures was tried. This did not prove successful since an envelope titre of 1 in 25 to 1 in 50 was still produced. The organism was heated at different temperatures in view of the observations of Schütze (1932) on the effect of heat on plague antigens. It was only after boiling the suspension for more than 15 minutes that it was possible to haptenize the envelope antigen completely. Such boiled suspensions proved to be quite effective in raising pure somatic sera.

The envelope antigen content of the virulent, the protective avirulent and the non-protective avirulent strains of Pasteurella pestis.

Reference was made in the study of morphology of the plague bacillus to certain quantitative differences in the envelope substance of the various types of *Pasteurella pestis* strains as determined by microscopic examination. This difference was reflected in the degree of opacity of the envelope halo round the organism and in its effect on their staining by aniline dyes. This observation was now tested by the application of agglutination and agglutinin-absorption methods.

Representative strains of the virulent, the protective avirulent and the non-protective avirulent groups were agglutinated against anti-plague sera before and

after their absorption by these strains. The results of one of these experiments are represented in Table IV :—

TABLE IV.

The agglutination of and the absorption by the virulent, the protective avirulent and the non-protective avirulent strains of Pasteurella pestis.

Agglutinating serum.	Dilution.	*PASTEURELLA PESTIS* STRAINS.		
		VIRULENT.	PROTECTIVE AVIRULENT.	NON-PROTECTIVE AVIRULENT.
		Preanger.	Tjiwidej 'smooth'.	'Q'.
Preanger ..	10	+++	+++	+++
	25	+++	+++	+++
	50	+++	+++	+++
	100	+++	+++	+++
	200	(±)	+++	+++
	500	—	—	+++
	1,000	—	—	—
	2,000	—	—	—
Preanger absorbed with Preanger.	10	—	—	—
	25	—	—	—
	50	—	—	—
	100	—	—	—
	200	—	—	—
	500	—	—	—
	1,000	—	—	—
	2,000	—	—	—
CONTROL ..		—	—	—

TABLE IV—*concld.*

Agglutinating serum.	Dilution.	*PASTEURELLA PESTIS* STRAINS.		
		VIRULENT.	PROTECTIVE AVIRULENT.	NON-PROTECTIVE AVIRULENT.
		Preanger.	Tjiwidej 'smooth'.	'Q'.
Preanger absorbed with Tjiwidej 'smooth'.	10	+++	+++	+++
	25	+++	+++	+++
	50	++	++	+++
	100	—	++	++
	200	—	—	++
	500	—	—	—
	1,000	—	—	—
	2,000	—	—	—
Preanger absorbed with 'Q'.	10	+++	+++	+++
	25	+++	+++	+++
	50	+++	+++	+++
	100	++	++	+++
	200	—	++	++
	500	—	—	++
	1,000	—	—	—
	2,000	—	—	—
CONTROL ..		—	—	—

It will be seen from Table IV that the intensity of envelope agglutination—represented by the interaction of serum 'Preanger' against strains 'Preanger', 'Tjiwidej smooth' and 'Q'—varies considerably with the three types of organisms, the difference being as much as five times. The fully virulent strain 'Preanger' reacting with its homologous serum gives a reading which is two and a half times lower than that obtained against the protective avirulent strain 'Tjiwidej smooth' and as much as five times less than that against a salt stable member of the non-protective avirulent group (strain 'Q'). In their study on the Vi antigen of

Salmonella typhi Felix, Bhatnagar and Pitt (1934) observed that with the strains of highest virulence the Vi titre of a serum was inversely proportional to the amount of Vi antigen possessed by the agglutinated organism—the higher the Vi content the lower the titre of reaction and vice versa. For this phenomenon these workers advanced an explanation that for the neutralization of a certain quantity of an antigen an equally proportionate amount of the antibody was required; where this proportion was high in an agglutination test a low reading was obtained and where it was low the amount of flocculation was high. It appeared that this theory may account for the variation in the degree of agglutinability exhibited by the three types of plague bacillus under consideration here if the results of absorption tests were to substantiate it. With this object in view, an identical amount of a plague antiserum was absorbed by an equal bulk of the three types of organisms under identical conditions. The results, which are included in Table IV, go to show that the largest quantity of the envelope antibody was absorbed by the virulent strain 'Preanger' and the least by the non-protective avirulent strain 'Q', the protective avirulent strain 'Tjiwidej smooth' occupying an intermediate position. It is thus clear that the capacity for envelope agglutinin absorption of these three strains and the intensity of their envelope agglutination are inversely proportional to each other. The quantitative difference in the amount of flocculation produced by the various strains of *Pasteurella pestis* against an anti-plague serum under identical conditions can, therefore, be explained in the same manner as agglutinating differences in the Vi containing virulent strains of *Salmonella typhi*.

It has been a common experience that the plague bacillus grown at 37°C. does not make a suitable agglutinating suspension. The titre of reaction against such a suspension is low and with weak sera it is apt to prove altogether inagglutinable (Pulgher, *loc. cit.*). From the experiments reported here it is evident that it is not the temperature of incubation which is responsible for these poor results but the fact that the importance of selecting the proper agglutinating agent in the light of the observations of Felix, Bhatnagar and Pitt (*loc. cit.*) on this subject has not yet been appreciated. For the maximum agglutination to take place it is essential that the organism employed for this purpose should be such that its surface antigen, or antigens, is neutralized by the minimum amount of corresponding antibody or antibodies. A normal plague bacillus contains a fairly large amount of the envelope antigen. Since the quantity of the envelope antibody in an anti-plague serum was never found to be very high, in spite of repeated courses of animal immunization, the plague bacillus as found in nature is not likely to make a suitable agglutinable suspension. Methods are, however, known by which the virulence of this organism can be reduced (Hetsch, 1904; Otten, 1936-37; Sokhey, 1932–35). It has already been shown by a study of the morphology of attenuated bacilli, whether classified as protective avirulents or as salt stable members of the non-protective avirulent group, that they are possessed of a lesser quantity of the envelope substance than the virulent strains of which they are the derivatives. Such strains should, therefore, be tested for their agglutinability as well as their salt and serum stability and one which is found to give the maximum degree of agglutination and to maintain this character over a long period should be utilized for the purpose of agglutination. Our non-protective avirulent strain 'Q' satisfies these criteria and

subcultured on 10 per cent horse-serum agar and incubated at 37°C. it has given reproducible results over a period of a year.

In his recent study on *Pasteurella pestis* Schütze (1939) suggested the possibility of their being another antigen apart from those already demonstrated, which may principally influence the prophylaxis in laboratory experiments on animals, especially the mouse. Methods which led to the discovery of the Vi antigen by Felix and his collaborators were applied to the various strains of the plague bacillus with this object in view. Apart from the quantitative difference in the amount of the envelope substance which these organisms were found to be capable of producing, no such factor has so far been encountered.

The serological inter-relationship of Pasteurella pseudotuberculosis *strains.*

Observations on the antigenic structure of the organism of pseudotuberculosis have been made by Schütze (1928, 1932) and by Kauffmann (1932). These authors concluded that the various strains of this organism were possessed of (*a*) a thermolabile flagellar 'H' antigen which is common to all the strains, and (*b*) multiple thermostable somatic 'O' antigens which are composed of factors certain of which are found in all the strains and others which are confined to a few strains only. On this basis the differentiation of *Pasteurella pseudotuberculosis* into various groups has been carried out. Schütze has associated the rough basal frame work of this organism with the possession of an antigen common to all the strains of *Pasteurella pseudotuberculosis* as well as that of *Pasteurella pestis*. To this antigen he has given the name of 'rough somatic antigen'.

(*a*) *Flagellar antigen.*—Arkwright (1927) has shown how the organism of pseudotuberculosis becomes motile, due to the development of the flagellar 'H' antigen, when grown at lower temperatures. The same phenomenon was noticed by us when the temperature of incubation was round about 22°C. The motility could not, however, be demonstrated when growth took place either at 37°C. or at 30°C. In the case of the plague bacillus no movement was observed at any temperature of incubation.

Sera were prepared from the growth at 22°C. of *Pasteurella pseudotuberculosis* and were subsequently absorbed by steamed suspensions of the homologous organism. It was observed that an antibody component was left in the serum after such absorption. This gave a woolly type of agglutination against all the groups and was entirely different in character from what has already been described as somatic agglutination of *Pasteurella pseudotuberculosis* by anti-plague sera.

(*b*) *Somatic antigens.*—The results of absorbing an anti-plague serum by various groups of *Pasteurella pseudotuberculosis* have already been described (*see* Table III). It was seen that in each case complete removal of that antibody which reacts with this organism took place. The various pseudotuberculosis sera were then absorbed by different *Pasteurella pestis* strains. The results of one of these experiments are reproduced in Table V. It may be stated here that the absorption of these sera either by the same strain or by different types of strains of the plague bacillus did not make any difference to the findings obtained.

TABLE V.

Agglutination by sera of various subgroups of Pasteurella pseudotuberculosis *after their absorption by different strains of* Pasteurella pestis.

Type of pseudotuberculosis subgroup serum.	Dilution.	*PASTEURELLA PSEUDOTUBERCULOSIS* STRAINS.				
		SUBGROUP I.		SUBGROUP II.		SUBGROUP III.
		IA.	IB.	IIA.	IIB.	
IA absorbed with *Pasteurella pestis* 'Preanger'.	10	+++	+++	–	–	–
	25	+++	+++	–	–	–
	50	++	++	–	–	–
	100	++	++	–	–	–
	200	–	–	–	–	–
	500	–	–	–	–	–
	1,000	–	–	–	–	–
IB absorbed with *Pasteurella pestis* 'Tjiwidej smooth'.	10	+++	+++	–	–	–
	25	+++	+++	–	–	–
	50	++	++	–	–	–
	100	++	++	–	–	–
	200	+	+	–	–	–
	500	–	–	–	–	–
	1,000	–	–	–	–	–
IIA absorbed with *Pasteurella pestis* 'E. V.'	10	–	–	+++	+++	–
	25	–	–	+++	++	–
	50	–	–	++	+	–
	100	–	–	++	–	–
	200	–	–	–	–	–
	500	–	–	–	–	–
	1,000	–	–	–	–	–
CONTROL ..		–	–	–	–	–

TABLE V—*concld.*

Type of pseudotuberculosis subgroup serum.	Dilution.	*PASTEURELLA PSEUDOTUBERCULOSIS* STRAINS.				
		SUBGROUP I.		SUBGROUP II.		SUBGROUP III.
		IA.	IB.	IIA.	IIB.	
IIB absorbed with *Pasteurella pestis* 'Q'.	10	—	—	+++	+++	—
	25	—	—	+++	+++	—
	50	—	—	+++	++	—
	100	—	—	++	+	—
	200	—	—	+	—	—
	500	—	—	—	—	—
	1,000	—	—	—	—	—
III absorbed with *Pasteurella pestis* 'I'.	10	—	—	—	—	+++
	25	—	—	—	—	+++
	50	—	—	—	—	+
	100	—	—	—	—	+
	200	—	—	—	—	—
	500	—	—	—	—	—
	1,000	—	—	—	—	—
CONTROL ..		—	—	—	—	—

It is evident from Table V that the plague bacillus is incapable of removing all the agglutinins present in a pseudotuberculosis serum. Before absorption all the sera reacted with all the group strains of *Pasteurella pseudotuberculosis.* After absorption the serum could only flocculate those strains which belonged to that group of the organism against which the serum was raised. This observation would indicate that besides the antigen common to it and the plague bacillus, *Pasteurella pseudotuberculosis* is possessed of antigens which are present only in certain strains of this organism.

The inter-relationship between different strains was further studied by reciprocal absorption of the sera already absorbed by the plague bacillus. The results are shown in Table VI :—

TABLE VI.

Agglutination by and cross absorption of sera of various subgroups of Pasteurella pseudotuberculosis.

Pseudotuberculosis subgroup serum.	Dilution.	*Pasteurella pseudotuberculosis* strains. Subgroup I. IA.	Subgroup I. IB.	Subgroup II. IIA.	Subgroup II. IIB.	Subgroup III.
IA absorbed with IB	10	+++	–	–	–	–
	25	++	–	–	–	–
	50	+	–	–	–	–
	100	–	–	–	–	–
	200	–	–	–	–	–
IB absorbed with IA	10	–	+++	–	–	–
	25	–	++	–	–	–
	50	–	–	–	–	–
	100	–	–	–	–	–
	200	–	–	–	–	–
IIA absorbed with IIB	10	–	–	+++	–	–
	25	–	–	++	–	–
	50	–	–	++	–	–
	100	–	–	–	–	–
	200	–	–	–	–	–
IIB absorbed with IIA	10	–	–	–	++	–
	25	–	–	–	++	–
	50	–	–	–	+	–
	100	–	–	–	–	–
	200	–	–	–	–	–
Control ..		–	–	–	–	–

It will be seen from Tables V and VI that after removal by the plague bacillus of that antibody common to all the strains of *Pasteurella pseudotuberculosis*, there are other components in the serum which are common only to the two subtypes of groups I and II of this organism. Reciprocal absorption of these sera revealed still another antibody which reacted only with the strain against which the serum was produced.

As a result of this study the various strains of *Pasteurella pseudotuberculosis* may be stated to be possessed of somatic 'O' antigens of the following order :—

(1) An antigen shared by all the strains of this organism as well as by *Pasteurella pestis*—the common group antigen.

(2) An antigen present only in certain strains of this organism—the group specific antigen.

(3) An antigen which characterizes individual strains—the type specific antigen.

The relationship of these findings to those of Schütze and of Kauffmann will form part of the general discussion.

Agglutination tests with killed suspensions of Pasteurella pestis *and* Pasteurella pseudotuberculosis.

In view of the objection to the use of live organisms the agglutinability of suspensions rendered sterile by heat and by the action of various chemicals was investigated. The opacity of the suspensions was the same as that recommended for live agglutination, namely, $1,000 \times 10^6$ organisms per c.c. for *Pasteurella pestis* and $1,500 \times 10^6$ organisms per c.c. for *Pasteurella pseudotuberculosis*, these two figures corresponding to Brown's opacity tubes Nos. 2 and 3, respectively. An exposure to 53°C. for half an hour was required to kill the bacilli. Any lowering of this temperature or lessening of the period resulted in the suspension not being always sterile. The chemicals selected for this purpose were silver nitrate (0·002 per cent), phenol (0·5 per cent), formalin (0·1 per cent), mercuric perchloride (1 in 10,000) and the extraction of organisms with alcohol. While silver-killed bacilli were prepared in the manner described by Rainsford (*loc. cit.*), in other cases the growth was washed with carbol saline, formol saline and mercuric saline of the requisite strength and later standardized to the opacity already mentioned. Alcohol extraction was carried out as is practised for the preparation of 'O' suspensions of *S. typhi* and *B. proteus*. The suspensions so prepared were stored for a month in the cold and their agglutinability tested every fourth day. The results obtained are exemplified in Table VII.

It will be seen from Table VII that the agglutinating activity of all the suspensions of *Pasteurella pestis*, whether killed by heat or by the use of a chemical, compared favourably with their live counterpart. Chemically sterilized suspensions of *Pasteurella pseudotuberculosis*, on the other hand, did not produce such satisfactory results, there being a marked lowering in the degree of this reaction with

TABLE VII.

Agglutination of killed suspensions of Pasteurella pestis *and* Pasteurella pseudotuberculosis.

Anti-plague serum.	Dilution.	*PASTEURELLA PESTIS* STRAIN 'Q'.							*PASTEURELLA PSEUDOTUBERCULOSIS* STRAIN 'PRII'.						
		Live.	Heated 53°C. ½ hour.	Formolized 0·1 per cent.	Carbolized 0·5 per cent.	Alcoholized.	Mercurialized ($HgCl_2$) 1 : 10,000.	Silver-killed 0·002 per cent ($AgNO_3$).	Live.	Heated 53°C. ½ hour.	Formolized 0·1 per cent.	Carbolized 0·5 per cent.	Alcoholized.	Mercurialized ($HgCl_2$) 1 : 10,000.	Silver-killed 0·002 per cent ($AgNO_3$).
E. V.' ..	25	+++	+++	+++	+++	+++	+++	+++	+++	+++	++±	+++	−	−	++
	50	+++	+++	++±	+++	+++	+++	+++	+++	+++	++	+−+	−	−	+±
	100	+++	+++	++±	+++	+++	+++	+++	+++	+++	+	+++	−	−	+
	200	+++	+++	++±	++±	+++	+++	+++	+++	+++	−	++	−	−	+
	500	+++	++±	++	+±	+++	+++	++	+++	+++	−	+	−	−	+
	1,000	+++	++	+	+	++	++	++	+++	++	−	+	−	−	−
	2,000	−	−	−	−	−	−	−	++	+±	−	−	−	−	−
	5,000	−	−	−	−	−	−	−	+	+	−	−	−	−	−
CONTROL ..		−	−	−	−	−	−	−	−	−	−	−	+++	++	−
Preanger ..	25	+++	+++	+++	+++	+++	+++	+++	+++	+++	++±	+++	−	−	+++
	50	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	−	−	++
	100	+++	+++	++±	+++	+++	+++	+++	+++	+++	−	+++	−	−	+
	200	+++	++±	++±	++	++	++±	++±	+++	++	−	++	−	−	+
	500	+++	++	++±	+±	+	+±	++	++	+	−	+	−	−	−
	1,000	−	−	−	−	−	−	−	+	±	−	+	−	−	−
	2,000	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	5,000	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CONTROL ..		−	−	−	−	−	−	−	−	−	−	−	+++	++	−

formolized, silver-killed and carbolized products while alcohol treated and mercurialized organisms did not prove to be stable in normal saline. Heat-killed bacilli gave better results with both the species but it was noticed that plague bacilli so treated became salt sensitive after a few days' storage.

It may, therefore, be concluded from the experiments described that to obtain reliable results in the study of the serology of plague and of pseudotuberculosis, the use of live organisms should be preferred for the purpose of agglutination. If, however, the employment of killed suspensions is desired, chemically treated plague bacilli and heat-killed organisms of pseudotuberculosis should be employed.

Summary.

1. The two difficulties in serological studies on *Pasteurella pestis* and *Pasteurella pseudotuberculosis*, namely, the preparation of stable suspensions and the production of immune sera of high agglutinating titres, have been overcome by a new method for agglutination tests and by a new procedure for the immunization of laboratory animals. The antigen-antibody reactions of the plague bacillus and the organism of pseudotuberculosis have been studied by the application of these methods as well as by agglutinin absorption.

2. An anti-plague serum agglutinates all the strains of *Pasteurella pseudotuberculosis* examined, thus indicating the presence of an antigen common to the two organisms. An anti-pseudotuberculosis serum, on the other hand, does not react with any of the strains of *Pasteurella pestis*. This anomaly is explained.

3. In an anti-plague serum the presence of two different antibodies has been demonstrated. One of these corresponds to the envelope of the organism—the envelope antibody—and the other to the soma of the bacterium—the somatic antibody. By the new method of agglutination the envelope and the somatic titres of a serum can be determined. The characteristics of the two types of agglutination differ.

4. A study of the serological inter-relationship between the different strains of *Pasteurella pseudotuberculosis* has been made. This indicates that this organism is possessed of at least three different antigens, namely (*a*) a common group antigen which is present in the plague bacillus as well, (*b*) a group specific antigen shared only by certain strains, and (*c*) a type specific antigen which characterizes individual strains.

5. The agglutinating activity of suspensions of *Pasteurella pestis* and *Pasteurella pseudotuberculosis* killed by heat and by the use of various chemicals is described.

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THE VIABILITY OF THE *MENINGOCOCCUS* IN THE COOL ROOM ($3\frac{1}{2}$°C. TO $8\frac{1}{2}$°C.).

BY

DHARMENDRA, M.B., B.S., D.B.

(*From the School of Tropical Medicine, Calcutta.*)

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THE *meningococcus* is extraordinarily sensitive to external influences and dies out very rapidly when removed from the body. Recently-isolated strains die out rapidly on culture media and subcultures have to be made every other day to maintain such strains. Under artificial cultivation the organism becomes more resistant and subcultures can be made at monthly intervals if suitable media are used.

The optimum temperature for growth is between 36°C. and 38°C., there being no growth below 30°C.

The optimum temperature for the storage of the meningococcus *culture.*—It is generally believed that the most suitable temperature for the maintenance of the grown *meningococcus* cultures is the incubator temperature (37°C.). The recent work of Pabst (1935) and others, however, goes to show that this may not be necessarily so. The work reported in this paper also does not support the belief that the incubator temperature is always the most suitable temperature for the maintenance of the grown *meningococcus* cultures. Before reporting the results a brief reference may be made to the known facts regarding the viability of the *meningococcus* cultures at temperatures lower than 37°C.

Room temperature.—Elser and Huntoon (1909) kept 15 strains on glucose agar in a dark room (25°C. to 26°C.) and found that 9 survived for 3 weeks and 6 for 4 weeks and that no strain could survive an exposure of 5 weeks. Murray (1929) found that the different strains varied in resistance, some dying in 4 days and the others living for several weeks.

Ice-chest temperature (6°C. to 8°C.).—Elser and Huntoon (*loc. cit.*) found the different strains surviving from 3 to 6 days, none survived for a week. Most of the other workers agree on this point but Bettencourt and Franca (Elser and Huntoon, *loc. cit.*) found that a number of strains remained alive for a month.

Temperature below freezing point.—Flexner (Pabst, *loc. cit.*) observed that thick suspensions of *meningococcus* survived for 5 days at —5°C. Von Lingelshiem (Murray, *loc. cit.*) found that *meningococcus* survived at —10°C. and —20°C. for short periods. Murray (*loc. cit.*) subjected small amounts of culture smeared on the sides of tubes to —63°C. and —78°C. for about 15 to 20 minutes. On subsequent subcultures he obtained quite good and rapid growth. Elser and Huntoon (Pabst, *loc. cit.*) stated that *meningococcus* might remain alive for years if dried rapidly under freezing temperatures and kept frozen. Swift (1921) by means of freezing and then drying the cultures in vacuum has reported the viability of *meningococci* after a period of at least 2 months. Rake (1935) has described a different technique for the freezing and drying of *meningococci* and has reported viability of *meningococci* after a period of at least 5 months.

Francis (1932) had reported the preservation of virulence of *Past. pestis* at —15°C. in a guinea-pig spleen suspended in pure undiluted neutral glycerine. Pabst (*loc. cit.*) applied this simple method to the maintenance of *meningococcus* cultures. He stored 10 strains of *meningococcus* in neutral glycerine at —15°C. for 2 years with no apparent change in viability, morphology, serological or biochemical characteristics. Another 223 strains were stored at this temperature on glucose-agar slants, both with and without glycerine with no appreciable loss in viability in the 8 months during which they had been under observation.

The present work.

The temperature of the inner chamber of the cool room at the School varies from 3½°C. to 8½°C. It was decided to ascertain the length of time for which *meningococci* remain viable at this temperature and to compare it with the time for which they remain viable at the incubator temperature (37°C.). Apart from the temperature at which the cultures are stored the viability of the *meningococcus* varies with the medium used and the duration for which the strains have been kept in the laboratory on artificial media. The strains used for these experiments had been grown on artificial media for practically the same length of time. In all 35 strains were used for the tests and two different media were used—pigeon-blood-agar slants and semi-solid serum-agar tubes.

Two sets of subcultures on pigeon-blood agar and serum agar were made and incubated for 48 hours. One set of each was then put in the cool room and the other in the incubator. Subcultures from these tubes were made every week and examined after incubating at 37°C. for 48 hours. The tube from which no growth could be obtained on two successive weeks was discarded and no further subcultures were made from it. The results of these observations till the end of 31 weeks are recorded in the Table (*see* pp. 47-48). It will be noticed that growth was obtained from all the tubes at the end of the first week and that the number of cultures found viable after subsequent weeks fell gradually. In the incubator no growth was obtainable from any of the tubes after 8 weeks in case of serum-agar cultures and after 10 weeks in case of pigeon-blood-agar cultures. In the cool room, on the other hand, while most of the strains lived for about 5 weeks 6 strains in each medium

survived for a much longer time. Growth was obtainable from these 6 cultures from serum-agar tubes till the end of 27 weeks and from pigeon-blood-agar tubes till the end of 31 weeks when last tested. These long-lived 6 cultures in both the media represented the same 6 strains and belonged to group II (Griffith). When the results in the two media are studied separately the following points are to be noted:—

PIGEON-BLOOD-AGAR CULTURES.

1. At the end of the 1st week all the 35 strains were recoverable both from the cool room and the incubator cultures. While all the subcultures from the cool-room culture tubes showed good growth, 11 subcultures from the incubator showed only scanty growth.

2. At the end of the 2nd week the difference in the viability of cool room and the incubator stored cultures was quite marked. Growth was obtained from all the 35 strains in the cool room but only 13 of the incubator cultures were recoverable. If the 6 strains which have proved to be especially hardy are excluded it will be noted that growth was obtained from all the 29 cultures kept in the cool room, while only 7 of the incubator cultures showed any growth.

3. At the end of the 3rd week growth was obtained from 23 tubes in the cool room and only from 8 tubes in the incubator. Excluding the 6 more resistant strains we find that out of the 29 strains, 17 were recoverable from the cool-room cultures at the end of 3 weeks, while only 3 could be recovered from amongst the incubator cultures.

4. At the end of 6 weeks while 10 strains were recoverable from the cool-room cultures, only 5 could be obtained from amongst the incubator cultures and all these 5 belonged to the 6 more resistant strains.

5. At the end of 10 weeks only 6 strains could be recovered from the cool-room cultures and 4 from the incubator. At the end of 11 weeks the same 6 strains were recoverable from the cool-room cultures and none from the incubator cultures.

6. When last tested, at the end of 31 weeks, these 6 cool-room cultures were still viable.

From the above observations it can be concluded that at the incubator temperature more than three-fourths of the cultures died at the end of 3 weeks, while at least two-thirds of them were alive at the end of 4 weeks in the cool room. If only the 6 more resistant strains are considered we find that all of them were recoverable from the cool-room cultures, when tested last at the end of 31 weeks, while in the incubator only 5 of them could be recovered at the end of 3 weeks, 4 at the end of 10 weeks, and none at the end of 11 weeks.

When the viability of the individual strains of the *meningococci* at the incubator and the cool-room temperature was considered it was noted that all the strains except two lived for a longer period in the cool room than in the incubator.

From these observations one can conclude that for storing cultures of *meningococcus* grown on pigeon-blood agar, cool-room temperature ($3\frac{1}{2}$°C. to $8\frac{1}{2}$°C.) is much better than the incubator temperature (37°C.).

Serum-agar culture.

1. At the end of 1st and 2nd weeks all the 35 strains were recoverable from both the cool-room and the incubator cultures. While good growth was obtained after 24 hours' incubation in case of subcultures from the incubator tubes, the subcultures from the cool-room tubes usually took 48 hours to show any growth and in most cases the growth was not profuse.

2. At the end of 3rd and 4th weeks more strains were recoverable from the incubator cultures than from the cool-room cultures.

3. At the end of 5 weeks 20 strains could be recovered from the incubator cultures and only 6 from the cool-room cultures. These 6 strains represented the more resisting strains already referred to. Excluding these 6 strains we find that at the end of 5 weeks 14 out of the 29 strains could be recovered from the incubator cultures, while none could be recovered from the cool-room cultures.

4. The 6 strains that survived the cool-room temperature for 5 weeks were found viable till the end of 27 weeks. None of these strains were recoverable from the incubator cultures after 7 weeks.

From these observations it can be concluded that as a rule incubator temperature is better than the cool-room temperature for storing *meningococcus* cultures grown in semi-solid serum-agar tubes. This is quite opposite to what we found in case of pigeon-blood-agar cultures. When we consider the 6 more resisting strains we find the cool-room temperature giving better results as in the case of pigeon-blood-agar cultures. All these 6 strains were found viable in the cool room at the end of 27 weeks, while in the incubator none could survive an exposure of 7 weeks.

It is difficult to explain the difference in the behaviour of the pigeon-blood-agar and the serum-agar cultures in the cool room.

Summary.

1. Thirty-five strains of *meningococci* have been studied as regards their viability at the incubator temperature and in the cool room ($3\frac{1}{2}$°C. to $8\frac{1}{2}$°C.). These strains had been maintained in the laboratory for some time before being tested.

2. Two different media have been used for these experiments, the pigeon-blood-agar slants and the semi-solid serum-agar media.

3. Six of these 35 strains have been found far more resistant than the others. All these 6 strains belonged to group II (Griffith) and survived in the cool room for 27 weeks in serum-agar medium and for at least 31 weeks on pigeon-blood-agar slants. None of the other strains survived for more than 8 weeks in either medium and at either temperature.

4. In case of pigeon-blood-agar cultures the cool-room temperature has been found to be much better for storing the grown cultures than the incubator temperature.

5. In case of serum-blood-agar cultures as a rule the incubator temperature was found to be better than the cool-room temperature. There was an exception in case of the 6 more resisting (group II) strains as they survived for a much longer time in the cool room than in the incubator.

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TABLE.

Showing the viability of the 35 *strains of* meningococci *in the cool room and at incubator temperature* (37°*C*.).

Time in weeks at the end of which sub-cultures made.	NUMBER OF STRAINS FOUND ALIVE.			
	PIGEON-BLOOD-AGAR CULTURES.		SERUM-AGAR CULTURES.	
	In the cool room.	In the incubator.	In the cool room.	In the incubator.
1	35	35	35	35
2	35	13	35	35
3	23	8	30	35
4	23	8	14	20
5	13	8	6	20
6	10	5	6	16
7	9	5	6	4
8	6	5	6	3
9	6	5	6	..
10	6	4	6	..
11	6	..	6	..

TABLE—*concld.*

Time in weeks at the end of which sub-cultures made.	NUMBER OF STRAINS FOUND ALIVE.			
	PIGEON-BLOOD-AGAR CULTURES.		SERUM-AGAR CULTURES.	
	In the cool room.	In the incubator.	In the cool room.	In the incubator.
12	6	..	6	..
13	6	..	6	..
14	6	..	6	..
15	6	..	6	..
16	6	..	6	..
17	6	..	6	..
18	6	..	6	..
19	6	..	6	..
20	6	..	6	..
21	6	..	6	..
22	6	..	6	..
23	6	..	6	..
24	6	..	6	..
25	6	..	6	..
26	6	..	6	..
27	6	..	6	..
28	6	..	..	..
29	6	..	..	..
30	6	..	..	..
31	6	..	..	..

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CLOSTRIDIUM BOTULINUM IN SAMPLES OF CALCUTTA SOIL.

BY

MAJOR C. L. PASRICHA, I.M.S.,
Professor of Bacteriology and Pathology,

AND

G. PANJA,
Assistant Professor of Bacteriology.

(From the Department of Bacteriology and Pathology, School of Tropical Medicine, Calcutta.)

[Received for publication, February 20, 1940.]

As a result of the extensive investigations of Meyer and his collaborators (1922) and others it has been shown that *Clostridium botulinum* is a common soil anærobe. It has been recovered from samples of soil from different parts of America, Canada, Europe, Africa, Australia, China and Hawaii but there is no record of any such search having been made of the soils from India.

Eight samples of surface garden soil were collected from certain open spaces in the city and examined for the presence of *Cl. botulinum.* The characteristic *botulinum* toxin was demonstrated in cultures made with four of the eight samples. Pure cultures of *Cl. botulinum* were isolated from the four positive samples.

The methods employed for the demonstration of the toxin and the isolation of the bacilli were based on those used by Dubovsky and Meyer (1922) and Wheeler and Humphreys (1924) and are summarized below :—

1. One level teaspoonful of the soil freed from coarse particles was suspended in 10 c.c. of saline (pH 7·0). The soil suspensions were neutral to litmus paper. In order to avoid the destruction of *Cl. botulinum* in the subsequent heating it is important that the reaction should be neutral. The suspensions can be neutralized with sodium carbonate if acid or dilute lactic acid if alkaline in reaction.

2. The soil suspension was heated in a water-bath at 60°C. for 1 hour.

3. Two cubic centimetres of the heated suspension were added to 20 c.c. of Hitchen's medium (0·2 per cent glucose infusion broth containing 0·1 per cent agar).

4. The inoculated tubes of Hitchen's medium were incubated under anærobic condition (McIntosh and Fildes' jar) for four days at room temperature (30°C. to 35°C.).

5. The growth in Hitchen's broth was heated at 70°C. for 1 hour.

6. Half to one cubic centimetre of the heated culture was inoculated in cooked-meat medium (in 8″ × 1″ tubes) and incubated anærobically for 15 days.

7. Toxicity tests. The following tests were done :—

(A) Two to three cubic centimetres of the clear fluid from the top of the culture in cooked-meat medium were injected subcutaneously into guinea-pigs. In order to avoid death due to tetanus toxin all animals used in these experiments received 300 units of tetanus antitoxin four hours before the injection of the suspected *botulinum* toxin. Subsequent experiments showed that fowls which are naturally immune to tetanus toxin can be used without the necessity of protection against tetanus.

(B) The cultures which caused the death of the animals were tested further as follows :—

Two to three cubic centimetres of the culture were injected into two series of animals, one of which had been protected with subcutaneous injections of 2 c.c. of *botulinum* antitoxic serum (Human A & B unconcentrated Burroughs Wellcome & Co.). As homologous type A and type B antitoxic sera were not available protection tests with the separate antitoxic sera could not be put up. A third series of animals which had received normal horse serum were also injected with the culture.

(C) The cultures which gave positive antitoxin test were further tested by feeding experiments. Series of protected (with *botulinum* antitoxic serum) and unprotected animals were given 3 c.c. to 4 c.c. of the culture directly into the stomach. This was conveniently given with a two-inch stout needle attached to a syringe. The end of the needle had been thickened with solder into a small olive-shaped globule. The feeding was easier when the animals had been starved overnight.

Other animals were given the filtrate which had been (*a*) heated at 100°C. for half an hour and (*b*) treated with 20 per cent alcohol for 30 minutes at room temperature.

All experiments were controlled with cultures or filtrates of cultures made with a laboratory strain of *Cl. botulinum* type A.

Four of the eight samples of the garden soil examined gave positive results. The animals injected with 2 c.c. to 3 c.c. of 15-day old culture in cooked-meat

medium died within 24 hours, whereas the animals that had been protected with *botulinum* antitoxic serum remained alive and well. Horse serum had no protective value. When cultures or filtrates of cultures of these samples were given by mouth to guinea-pigs the animals died within 24 hours. Animals that had been protected with antitoxic serum remained well. Animals that were given heated filtrates (heated at 100°C. for half an hour) or cultures treated with alcohol remained well.

Post-mortem examination was made of all the animals. In addition to the findings described by Topley and Wilson (1936) there was present in the lungs (more marked in the right lung) of all animals dark red areas which became bright red on exposure. Microscopically there was well-marked dilatation of the capillaries in the walls of the alveoli. The suprarenals were pale pink and microscopically showed congestion of the medulla. Similar changes were noted in animals that died after injection of filtrates of a culture made with the laboratory strain of *Cl. botulinum.*

Pure strains of *Cl. botulinum* were isolated from all the four cooked-meat broth soil cultures. The culture in cooked-meat broth was heated at 80°C. for half an hour and then plated on horse blood (5 per cent), glucose (½ per cent), and nutrient agar (2 per cent). Suspicious colonies were picked up. The final identification was made by testing the toxigenic property of each colony.

Smears made from a four-day old growth at 37°C. on agar show large stout bacilli with rounded ends, straight axis, parallel sides, occurring singly, sometimes in pairs or short chains. A few filamentous forms are present in some of the smears. The spores are oval in shape, subterminal in position and cause distinct swelling of the bacilli. Many free spores are present. The organism is sluggishly motile. The motility can be seen in film preparation made from the growth in cooked-meat medium, and the growth in semi-solid agar is of the penetrating spreading type seen with motile bacilli. The organisms retain the Gram's stain. There are no capsules. The organism grows well on ordinary media under anærobic conditions. There was no apparent improvement in growth in jars to which 10 per cent CO_2 had been added.

After four days' incubation at 37°C. the colonies on nutrient-agar plate are irregularly round, 3 mm. to 4 mm. in diameter, shining, opaque, flat with a somewhat uneven surface and fimbriated edge. By reflected light the colonies are whitish yellow in colour. The centre of the colony is more opaque than the edge. The consistency is butyrous and the colonies are easily emulsifiable in water. In some plates two types of colonies are found, a small disc-shaped semi-transparent colony about 1 mm. in diameter without a spreading edge and the other colony as described above.

On blood agar after four days' incubation at 37°C. the colonies are larger, 1 cm. to 2 cm. in diameter, shining greyish white in colour, hyaline in structure with a transparent centre, and a thicker and slightly raised rolled-over periphery. There is clear hæmolysis around the colony and the colour of the blood is light brown.

In broth after five days at 37°C. the growth is confined mainly to the bottom of the tube, whereas in peptone water there is diffuse turbidity, with a slight woolly deposit which on shaking breaks up readily. There is no pellicle formation.

On agar slope there is confluent spreading growth on the lower three-fourths of the slope with a few isolated colonies at the top dry portion.

There is marked liquefaction of Loeffler's serum. On coagulated egg after four days' incubation at 37°C. the growth is moderate and the egg is partly digested. There is no discoloration of the medium.

In cooked-meat medium after four days' incubation at 37°C. there is marked turbidity with partial digestion and darkening of the meat. In all media there is an unpleasant rancid odour. This is most marked in cultures in cooked-meat medium.

In glucose-agar shake there is abundant gas formation with breaking up of the medium. Discrete tiny colonies with opaque brown centres are scattered throughout the medium.

The four strains produce acid and gas in glucose, maltose and salicin and do not ferment lactose, saccharose, dulcitol and mannitol. Litmus milk is coagulated and acidified at first and then liquefied in four days. The Voges-Proskauer and the methyl-red tests are both negative, the indole reaction is weakly positive. The spores are not killed by heating at 100°C. for 1 hour.

The toxins formed by this organism killed mice, rats, guinea-pigs, chickens, pigeons, sparrows, cats and dogs. A dose of 0·001 c.c. of the filtrate of a 15-day old culture in cooked-meat medium when injected subcutaneously killed the smaller laboratory animals such as mice, rats, guinea-pigs and chickens within 24 hours. Some of the toxins were doubtless lethal to guinea-pigs in smaller amounts but the minimum lethal dose was not determined. Much larger doses (4 c.c. to 5 c.c.) were injected into cats and dogs. When given by the mouth the toxin killed guinea-pigs, chickens and cats, the other animals were not so tested. Larger doses (2 c.c. to 8 c.c.) were used for the feeding experiments. That these doses were excessive was demonstrated by feeding healthy rat with the uncooked flesh and liver of a chicken that had died three days after having been fed with 2 c.c. of a toxic filtrate. The rat so fed died eight days later.

Cats injected subcutaneously with 4 c.c. of an 18-day old culture died in 3 to 4 days, whereas the cats which had received the same dose of toxin by mouth died in 8 to 9 days. There was marked weakness, listlessness and diarrhœa leading gradually to death. Their mental condition remained apparently normal.

Four dogs were injected subcutaneously with 5 c.c. of the unfiltered toxin from the four strains isolated. The dogs became listless, there was incontinence of urine, protrusion of the tongue and feeble efforts at barking and convulsions just before death, which occurred 18 to 60 hours after the injection of the toxin.

Post mortem both in cats and dogs there were hæmorrhagic patches in the lungs, more marked in the right lung, the right heart was dilated and the veins distended with blood. In one dog hæmorrhagic exudate was present throughout the whole of the intestines.

Similar results were obtained with filtrates of a culture of the laboratory strain of *Cl. botulinum* type A. The reasons for regarding the strains of *Cl. botulinum* isolated from the soils of Calcutta as type A may be summarized as follows:—

1. Biochemically type A is known to ferment salicin, whereas type B does not.

2. This toxin kills chickens when fed orally, whereas type B is stated to have no effect.

3. Type A toxin when injected subcutaneously kills dogs, whereas dogs are stated to be refractory to type B toxin. It was not possible to test this experimentally as type B strain of *Cl. botulinum* was not available.

During the course of this work two interesting points were noted. These organisms which are strict anærobes and will not grow on ordinary laboratory media when incubated ærobically, grow well ærobically when inoculated in blood clot or in mashed cabbage medium (a buffered mash of cabbage packed for about 3 inches in broth). Human blood clot was used and the depth of the clot varied from 1 to 2 inches. There was good growth in both the blood clot (which was liquefied and became port wine in colour) and in the cabbage mash medium. Potent toxin was formed in both these media under ærobic conditions, when tested after incubation for 14 days. The toxin of this organism is stated to be formed only under strict anærobic conditions but under experimental conditions it has been repeatedly found that when inoculated into cooked vegetables a highly potent toxin is formed. The vegetable mash was pushed up to the top of the fluid but there was no obvious change in the colour of the mash. There was a strong rancid odour. This would explain the rare cases of botulism occurring after the eating of stale food cooked in the house such as the one recorded in the *Lancet* (Annotation, 1935).

Preliminary experiments designed to repeat the work of Stark, Sherman and Stark (1928) in which they found that the addition of filtrates of *botulinum* culture to sterilized skimmed milk considerably increased the toxin content gave negative results.

Summary.

Cl. botulinum has been recovered from samples of soil from different parts of America, Canada, Europe, Africa, Australia and China but there is no record of any search having been made of the soils from India. Eight samples of garden soil were collected from certain open spaces in the city and examined for the presence of *Cl. botulinum*. The characteristic botulinum toxin was demonstrated in cultures made with four of the eight samples. Pure cultures of *Cl. botulinum* were isolated from the four positive samples. It was not thought necessary to extend this investigation. A study was made of the toxin elaborated by the four strains of *Cl. botulinum* and in all four the toxin behaved like *botulinum* toxin type A.

Although botulism is rare in India, only one outbreak of suspected botulism having been recorded by Torpy (1938), it is possible that with increasing use of tinned or preserved foodstuffs which have not been adequately handled there will be danger of further outbreaks.

During the course of this work an interesting observation was made that *Cl. botulinum* which is a strict anærobe grows well in blood clot under ærobic conditions. This work is being extended.

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THE OCCURRENCE OF THE *ÆROGENES* GROUP OF *COLIFORM* ORGANISMS IN FÆCES AND ITS SIGNIFICANCE IN WATER ANALYSIS.

BY

RAO SAHIB T. N. S. RAGHAVACHARI,

AND

P. V. SEETHARAMA IYER.

(*From the King Institute of Preventive Medicine, Guindy, Madras.*)

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THE conclusion was reached in a previous paper (Raghavachari and Seetharama Iyer, 1939) that the MacConkey's test at 44°C. outlined in the Medical Research Council Report, Series No. 206, can be considered specific for differentiating *coli* into fæcal and non-fæcal types, *only* if it can be proved that *ærogenes*-like organisms in water capable of growing at 44°C. are normal intestinal organisms in India. The present note gives an account of the subsequent work carried out on *coliform* bacteria isolated from fresh samples of human fæces from healthy human adults.

Before recording our results and findings, it is considered desirable to review the recent literature on the subject.

Mollari, Randall and Reedy (1939) carried out experiments to determine the incidence of *B. ærogenes* and intermediates in the fæces of human adults when single fæcal specimens were examined at different times of the year and isolated *B. ærogenes* from 40 per cent of the human adults examined in Washington, D. C.

Minkevich and Rabinovich (1936) are of the opinion that *B. coli* from the intestinal tract on coming into contact with soil adapts itself by changes in biochemical properties, particularly by increase in the utilization of citrate and in the splitting of saccharose and that all soil strains of *B. coli* are, therefore, evolved from fæcal *B. coli*.

Horwood and Webster (1937) studied seven samples of the ileostomy discharge and four of the rectal discharge from a patient taken during a period of eleven

months for their content of *coliform* organisms. From the results obtained they are inclined to believe that 'the normal bacterial flora in the small intestine consists essentially of *ærogenes* types but that following the passage of the contents from the small to the large intestines, a change occurs which transforms the *ærogenes* into *B. coli*. It is possible, therefore, when this transformation is not complete, that the *ærogenes* types have a much greater sanitary significance than is accorded to either type at present'. Obviously, therefore, these authors consider that *ærogenes* organisms are all of intestinal origin, being the normal inhabitants of the small intestine.

Parr (1937) in a study of the fæces of 201 human subjects by direct plating found 25·2 per cent of the organisms to belong to the *ærogenes* type. He also observed that the fæcal flora of the healthy subject is subject to such great variation that a number of points in sanitary, systematic and pathologic bacteriology may have to be restated.

Sen (1937) working in Calcutta investigated the biochemical reactions of lactose fermenters isolated from human fæces and compared the value of the methyl-red, V. P., citrate and indole tests with that of the Eijkman test at 44°C. He found that about 9 per cent of the organisms conformed to the *ærogenes* type and a considerable number of these also gave the positive Eijkman reaction.

The significance to be attached to the *ærogenes* group in sanitary water analysis would, therefore, appear to require re-assessment on the basis of further elaborate research, which should include controlled studies by different workers of fæces derived from both the sick and the healthy.

The experiments to be described were directed, among other things, to the elucidation of one particular point relating to the *ærogenes* organisms isolated from fæces, viz., if they will grow producing acid and gas in MacConkey's broth when incubated at a constant temperature of 44°C. in a water-bath.

Fresh specimens of fæces from fifteen apparently healthy individuals—members of the staff of the King Institute of Preventive Medicine—were obtained and tested by (1) direct plating, and (2) enrichment in MacConkey's bile-salt neutral-red lactose broth, at 37°C. and at 44°C.

1. *Direct plating.*—Each specimen was plated on two sets of MacConkey's bile-salt agar plates, one set was incubated at 37°C. and the other at 44°C., in electrically heated incubators for twenty-four hours. Ten pink colonies were picked out from each series and confirmed for lactose fermentation in MacConkey's single-strength broth tubes at 37°C. All strains which were confirmed were submitted to the following differential tests :—

(*a*) Fermentation reactions in saccharose, dulcite, adonite and inulin ;

(*b*) Biochemical reactions as judged by the methyl-red, V. P., citrate and indole tests ;

(*c*) Lactose fermentation in MacConkey's broth at a constant temperature of 44°C. in a water-bath.

2. *Enrichment method.*—One gramme of each specimen of fæces was emulsified in peptone water and 1 c.c. of the emulsion was added to each of two MacConkey's broth tubes (single strength). One was incubated at 37°C. in an ordinary incubator and the other at 44°C. in the water-bath for 24 hours. MacConkey's plate cultures were then made from each of the two broth cultures (37°C. and 44°C.) and the two plates were then incubated at 37°C. and 44°C. respectively for 24 hours at the end of which ten colonies were picked off from each plate and confirmed for lactose fermentation in MacConkey's broth at 37°C. The positive strains were then subjected to the same differential tests as those outlined above under 'direct plating'.

Table I gives the classification of the organisms isolated by the different methods into *coli*, *ærogenes-cloacæ*, intermediates and irregulars:—

TABLE I.

	Incubation at 37°C.		Incubation at 44°C.	
	Direct plating.	Enrichment.	Direct plating.	Enrichment.
Total number of organisms isolated.	113	128	109	128
Coli	80·5 per cent.	93·0 per cent.	100·0 per cent.	89·8 per cent.
A. C.	13·3 ,,	5·4 ,,	*Nil*	7·8 ,,
Intermediates ..	0·9 ,,	1·6 ,,	*Nil*	*Nil.*
Irregulars ..	5·3 ,,	*Nil*	*Nil*	2·4 per cent.

It will be seen that all the organisms obtained by direct plating in the 44°C. (incubator) series were true *B. coli,* while by the enrichment method (water-bath) in the same series, 7·8 per cent of the organisms belonged to *ærogenes-cloacæ* and 2·4 per cent to the irregular group. Direct plating followed by incubation at 37°C. yielded 13·3 per cent of *ærogenes-cloacæ* while the enrichment method at this temperature gave 5·4 per cent of *ærogenes-cloacæ* organisms.

Out of a total of 478 organisms studied, 31 belonged to the *ærogenes-cloacæ* group, 24 of these were capable of growing in MacConkey's broth at a constant temperature of 44°C. in a water-bath. Table II gives the

classification of the organisms on the basis of their ability to grow at 44°C. in a water-bath :—

TABLE II.

Type of organism.	DIRECT PLATING.						ENRICHMENT.					
	Incubated 37°C.			Incubated 44°C.			Incubated 37°C.			Incubated 44°C.		
	Total.	44°C. +	44°C. −	Total.	44°C. +	44°C. −	Total.	44°C. +	44°C. −	Total.	44°C. +	44°C. −
Coli I ..	81	78	3	103	102	1	116	116	0	111	104	7
Coli II ..	9	5	4	5	2	3	3	2	1	4	4	0
A. C. I ..	15	8	7	..	..	..	6	6	0	10	10	0
A. C. II ..	..	..	..	..	..	..	1	0	1	..	..	..
Inter. I ..	..	..	..	..	..	..	2	1	1	..	..	..
Inter. II ..	1	1	..	..	..	..	..	..	..	..	..	..
Irregulars	6	6	0	..	..	..	..	..	..	..	..	..

Eleven out of 411 organisms of the *coli* I group failed, on repeated trials, to grow at 44°C. The 24 strains of ærogenes which grew at 44°C. were subcultured from time to time, subjected to the 44°C. test every week for over 2 months, and found to be consistently positive all the time. Similarly, the seven strains which were initially negative, continued to be so during this period. Twenty-seven of the 31 strains comprised in the *ærogenes-cloacæ* I group, were either *B. lactis ærogenes* or *B.* 67 of the MacConkey's scheme and four were *B. cloacæ.*

Discussion.

The work of the past 30 years has tended to show that amongst the lactose-fermenting organisms isolated from water, a distinction can be made between the typical *B. coli* and the *B. ærogenes.* The latter has generally been considered to be non-fæcal in origin being derived from the soil and, therefore, of comparatively little or no sanitary significance in water analysis. From the review of the recent literature recorded in this paper and from our own results of the present study, a

revision of our ideas about the significance of the *ærogenes* groups would, however, appear to be indicated. Thirty-one strains out of a total of 478 lactose fermenters isolated from normal fæces (6·5 per cent) belonged to the *ærogenes-cloacæ* group I of the Ministry of Health classification scheme. Twenty-four or 80 per cent of these grew consistently in MacConkey's broth at a constant temperature of 44°C. in a water-bath while the remaining 7 which were otherwise identical with the other 24, consistently refused to grow at 44°C. Both these sub-groups maintained their power to grow or not at 44°C. even after repeated subcultures over a period of over 2 months. The question naturally arises, as to whether these two sub-groups of the *ærogenes-cloacæ* are really two different species or if those which fail to grow at 44°C. represent mutants or variants of those which grow at 44°C., the property of growth at 44°C. having been lost during their sojourn on land when removed from their normal (is it the normal ?) habitat in the human intestine ? The theory has been advanced that all *coliform* bacteria, wherever found, are fæcal in origin but lose certain biochemical and other properties and acquire certain others when removed from their normal habitat. Another hypothesis which, however, lacks adequate experimental confirmation, lays down that the bacterial flora of the small intestine is essentially all of the *ærogenes* group which undergoes a transformation (a remarkably quick one it would have to be) into the *B. coli* group when the large intestine is reached. When it is also remembered that a very large percentage of the *coliform* organisms isolated from pathological material, e.g., urine, blood, etc., is of the *ærogenes* group the necessity for an attempt at a revision in our estimate of the significance of the *ærogenes* group will be readily apparent. A considerable amount of further work will, however, be necessary, particularly in the tropics, before the real status of this group can be determined. The application of the 44°C. MacConkey's test as a routine measure along with the other four biochemical tests will have to be continued over a series of samples of water, fæces, milk, soil and foods collected from different sources and from different parts of the country, under varying seasonal and other limiting conditions.

Summary and conclusions.

1. Specimens of fresh fæces from fifteen healthy human subjects were examined by direct plating and by enrichment in MacConkey's media. In both cases, the tests were run in duplicate to permit of incubation at 37°C. and 44°C., respectively.

2. The direct plating followed by incubation in an incubator at 44°C. yielded lactose fermenters which proved to be all true *B. coli*, while on enrichment at the same temperature (in a water-bath) nearly 8 per cent of the organisms isolated belonged to the *ærogenes-cloacæ* group.

3. Direct plating followed by incubation at 37°C. gave 13·3 per cent of the *A–C* group, while enrichment at the same temperature gave only 5·4 per cent of the *A–C* organisms.

4. About 7 per cent of the total number of lactose fermenters belonged to the *ærogenes-cloacæ* group.

5. Nearly 80 per cent of the *ærogenes* organisms isolated consistently grew at 44°C. even on repeated subculture.

6. About 3 per cent of the *coli* I group type of organisms failed to grow at 44°C.

7. Sixty per cent of the specimens of fæces examined showed the presence of *ærogenes*.

The normal flora of human fæces contains members of the *ærogenes-cloacæ* group to the extent of about 7 per cent, about 80 per cent of which consistently give a clear positive reaction to the 44°C. MacConkey's broth test. A small number (3 per cent) of the *coli* I group gives a negative reaction to the same test.

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ATTEMPTS AT TRANSMISSION OF HUMAN LEPROSY TO SYRIAN HAMSTERS.

BY

DHARMENDRA, M.B., B.S., D.B.,
Junior Research Worker, Indian Research Fund Association,

AND

J. LOWE, M.D.,
Research Worker, British Empire Leprosy Relief Association (Indian Council).

(From the School of Tropical Medicine, Calcutta.)

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Introduction.

Mycobacterium lepræ was one of the first pathogenic organisms to be seen and described in the human tissues. Its discovery preceded that of the tubercle bacillus by about ten years. The discovery of the tubercle bacillus was of course followed by success in culture and animal inoculation, and by great advances in knowledge of the ætiology of the disease. The discovery of the leprosy bacillus has not so far been followed by similar success. It is doubtful if any culture has been obtained or any animal infected. Hope was aroused by the communication of Adler (1937) reporting that in Syrian hamster he had after all found an animal susceptible to human leprosy.

Adler's work.

Adler inoculated three splenectomized hamsters by implanting small pieces of leprous nodule under the skin of the abdomen, the inoculation being supplemented by intraperitoneal injection of 'macerated leprous material'. A fourth animal was inoculated by putting a small piece of the nodule in the muscles of the left thigh. A week after inoculation, one animal of the first batch was found dead

and undergoing putrefaction and was not, therefore, examined. Adler's observations are based on the findings in the other three animals. One of these three animals was sacrificed a week after inoculation, as it was found ailing, and the other two were sacrificed six weeks after inoculation. In the two animals in which a piece of nodule had been implanted under the skin of the abdomen, fibrous nodules about 1 cm. in diameter were found at the site of inoculation, and smears taken from these nodules 'contained innumerable lepra bacilli'. In one of these two animals (in the one sacrificed a week after inoculation) 'lepra bacilli' were, in addition, found in smears from liver, skin abrasions, and at the site of a previous operation performed for reasons not connected with the experiment. No trace of the implanted nodule was found in the animal inoculated in the left thigh but the regional lymphatic gland was found enlarged and 'caseating in the centre, and smears showed innumerable leprosy bacilli'. These findings were interpreted by Adler as indicating the susceptibility of the hamster to human leprosy.

Burnet (1938), following up Adler's work, put small fragments of nodule under the skin of six hamsters, three having been splenectomized. Four months later they were re-inoculated by intraperitoneal injection through a fine trocar. Four months later still they were sacrificed. Five showed nothing. The sixth hamster showed 'a subcutaneous lesion with the structure of an organized leproma' *at the site of inoculation and 'the beginning of generalization in the lymph nodes, liver,* kidneys and spleen'. Burnet discusses possible objections that the local lesion might have been merely a graft of human leproma with possible multiplication of bacilli in it, and that the bacilli found elsewhere might have been carried mechanically by lymph or wandering cells, from the original focus. His chief answer to these objections is that, if they were valid, similar findings should have been made in more or all of the animals and not merely in one.

Present work.

Twenty-six Syrian hamsters were inoculated with human leprosy material. The experiment was made at two different places: one batch of animals was inoculated and kept at Calcutta, and the other at Kasauli. The findings in both these batches have been similar; the results, therefore, will be considered together. Of the twenty-six animals inoculated, two escaped and were lost and one was not examined thoroughly as it was found dead and putrefied. The findings in only twenty-three animals are, therefore, reported.

The inoculum.—The material used for both the batches consisted of pieces of nodule taken from the ears of patients. Smears from these nodules showed a large number of leprosy bacilli. At Calcutta the material was used within two hours of its removal from the body of the patient, but at Kasauli it was impossible to use material until four hours after its removal.

Note.—One of us (J. L.) had an opportunity of seeing Adler's sections, and the appearance strongly suggested multiplication of bacilli in the tissues examined. The work here reported was done by the other of us (D.).

Treatment of animals previous to inoculation.—In thirteen of the twenty-three animals the spleen was removed three or four days previous to the implantation. In the other ten this was not done.

Methods of inoculation.—Two different methods of inoculation were used: insertion of a small piece of leprosy nodule under the skin, and intraperitoneal injection of an emulsion prepared from the nodule. The animals were divided into two groups; in one group the inoculation was made by the first route and in the other by the second route. Both these groups included splenectomized and non-splenectomized animals; the constitution of these groups is shown in Table I:—

TABLE I.

Methods of inoculation.

Mode of inoculation.	Number of animals.		
	Splenectomized.	Non-splenectomized.	Totals.
Intraperitoneal ..	6	4	10
Nodule under the skin ..	7	6	13
Totals ..	13	10	23

Period of observation.—As leprosy is a very slowly progressing disease, it was decided to let the animals live for a reasonably long time before they were sacrificed and examined. Six of the animals, however, died within three weeks after inoculation as a result of an accident (exposure to sun). One animal died six and a half months, and another about eight months, after inoculation. Of the remaining animals, twelve were sacrificed nine and a half months, and three one year, after inoculation.

Examinations made.—When an animal died or was killed, it was first examined for macroscopic lesions. Smears were then made from various tissues, a small piece of these tissues being removed for section and microscopical examination. The smears and sections were stained by Ziehl-Neelsen's method and were examined for acid-fast bacilli. In animals dying early, smears and sections were taken from

the implanted nodule, regional lymphatic gland, omentum, liver, spleen and lung only. In the animals killed nine months or more after inoculation, these examinations were made in a larger number of tissues—the implanted nodule, both inguinal glands, both axillary glands, cervical glands, omentum, liver, lung, kidney, suprarenal, spleen and mesenteric glands.

Findings.—Table II shows the findings made in the two groups of animals —splenectomized and non-splenectomized. As splenectomy does not appear to influence the results in any way, the findings in both the groups may be considered together.

TABLE II.

Findings in 23 Syrian hamsters inoculated with human leprosy material.

Mode of inoculation.	SPLENECTOMIZED.			NON-SPLENECTOMIZED.		
	RESULTS.			RESULTS.		
	Total number of cases.	Time for which the animal lived.	Findings.	Total number of cases.	Time for which the animal lived.	Findings.
Intraperitoneal	6	13 days	A few acid-fast bacilli in omentum. Nothing elsewhere.	4	9½ months	No acid-fast bacilli anywhere.
		13 days	No acid-fast bacilli anywhere.		9½ months	do.
		9½ months	do.		9½ months	do.
		9½ months	do.			
		9½ months	do.		1 year	do.
		9½ months	A few acid-fast bacilli in left inguinal gland. Nothing elsewhere.			

TABLE II—*concld.*

Mode of inoculation.	SPLENECTOMIZED.			NON-SPLENECTOMIZED.		
	RESULTS.			RESULTS.		
	Total number of cases.	Time for which the animal lived.	Findings.	Total number of cases.	Time for which the animal lived.	Findings.
Implantation of nodule.	7	17 days	Nodule—Large number of acid-fast bacilli. Left inguinal gland. Large number of acid-fast bacilli. Nothing elsewhere.	6	13 days	Nodule. Large number of acid-fast bacilli. Right inguinal gland. A few acid-fast bacilli. Nothing elsewhere.
		6½ months	Nodule. Large number of acid-fast bacilli. Nothing elsewhere.		13 days	Nodule. Large number of acid-fast bacilli. Nothing elsewhere.
		9½ months	do.			
		9½ months	do.		13 days	do.
		9½ months	Nodule not found. No acid-fast bacilli anywhere.		7¾ months	Nodule not found. No acid-fast bacilli anywhere.
		1 year	do.		9½ months	Nodule. Large number of acid-fast bacilli. Nothing elsewhere.
		1 year	do.		9½ months	do.

The findings reported in Table II may be briefly summarized as follows :—

(*a*) *Intraperitoneal injection*—10 *animals.*

No macroscopic lesion was observed anywhere in any of the animals.

No acid-fast bacilli were found on microscopic examination in eight of the ten animals. In the remaining two a few acid-fast bacilli were found, in smears from omentum in one case, and from an inguinal gland in another. The animal which showed bacilli in the omentum was one out of two examined only thirteen days after inoculation, the other positive finding being in one of eight examined nine months or more later. It appears therefore that bacilli may be more frequently found early than late in such an experiment.

(*b*) *Implantation of nodule*—13 *animals.*

Small masses were found at the site of implantation in nine of the thirteen animals. These masses were not appreciably bigger than the pieces of nodule which had been implanted; they were loosely attached to the surrounding connective tissue. Smears and sections from these masses showed very large numbers of acid-fast bacilli. We consider these masses to represent the originally implanted pieces of nodule.

In the remaining four animals the original implant could not be traced, nor were any lesions or bacilli found elsewhere. The following remarks refer to the other nine animals :—

Local lymph nodes.—In two of the nine animals the regional lymph glands were found enlarged and congested. These two animals had died within three weeks of inoculation. Smears from these enlarged glands showed a large number of acid-fast bacilli. No enlargement or bacilli were found in the glands of animals examined later in the experiment.

Other tissues.—In none of the nine animals were any lesions (macroscopic or microscopic) or acid-fast bacilli found in any site other than the site of original implant and the local lymph glands above referred to.

Interpretation of the above findings.—There is one important fact which must be remembered in discussing infection of animals with human leprosy bacilli, namely, that even dead bacilli have extraordinary powers of persistence in tissues of the living animal. This is well recognized by many workers. We ourselves have found slight lesions and many bacilli in rats examined one year or more after being inoculated with human leprosy bacilli killed by heat. In such animals bacilli are numerous to begin with and then get fewer as time goes on, but they may still be found many months later, not only at the site of inoculation but in the local lymph glands, the spleen, the liver and elsewhere. In interpreting results of animal inoculation care must be exercised in order to avoid the mistake of taking the presence of these lesions and bacilli as evidence of a progressive infection.

We have therefore examined our results critically with this fallacy in mind.

The only macroscopic lesions, apart from the implanted nodule, were found in the local lymph nodes. Such a finding was made in only two of the twenty-three animals, and both these animals had died within three weeks of inoculation.

Lepra bacilli were still very numerous in the implanted nodule after one year. It is difficult to say whether there was any multiplication of the bacilli in the implanted tissue, for bacilli found a year afterwards may be the original bacilli persisting, and we do not know whether they are living or dead

The only places other than the implanted nodules, where acid-fast bacilli have been found, are the omentum (in one animal) and the local lymph glands (in three animals). The circumstances in which these four positive findings were made were as follows :—

Three were made in six animals examined within three weeks, while the remaining one was made in seventeen animals examined nine months or more later.

Thus, bacilli were more often found early than late in the experiment. This fact suggests that bacilli found outside the implanted nodule had come from the original nodule and, possibly without any multiplication, were dealt with by the defensive forces of the body.

We have thus obtained no macroscopic or microscopic evidence of a generalized infection in Syrian hamsters following on inoculation of leprous material by either of the two methods described. We are not in a position to say whether any multiplication of bacilli occurs in the implanted nodule.

Discussion.

We have not been able to confirm the findings of Adler and of Burnet regarding the susceptibility of Syrian hamsters to human leprosy. We may briefly discuss some of the possible explanations of our failure.

Burnet has stated that the susceptibility of individual hamsters varies markedly and that a number of animals may have to be inoculated before one susceptible animal is found. It may be argued that we failed to infect the hamsters because none of the twenty-three animals in our series was a susceptible one. If that is so, it means that the susceptibility of hamsters to human leprosy is not of such an order as to make it of any real value in the study of the disease.

Another possible explanation is that the susceptibility of hamsters to leprosy may vary in different circumstances. In this connection it is interesting to note that some workers in India have found that the guinea-pig is less susceptible to experimental infection with human tuberculosis than it is in other countries.

Still another possibility is that other workers may not have made sufficient allowance for lesions produced by injected bacilli persisting for a long time without multiplication. Adler's experiment was a short one, lasting only six weeks, and the evidence he produces of the susceptibility of the hamster is based mainly on the findings in one animal which was sacrificed one week after inoculation. The animals sacrificed later showed fewer bacilli and lesions. It appears possible that these lesions were produced by the great number of bacilli in the original inoculum, for nodules and emulsions made from them usually contain enormous numbers of

bacilli. It is possible that if Adler had kept his animals for nine months or more, he would have obtained results similar to ours.

Burnet's experiment was a longer one than Adler's, the animals being kept for eight months after the original inoculation, but after four months an intraperitoneal injection (presumably massive) was given through a trocar. Although we consider it would have been better to keep the animals for more than four months after the last inoculation, Burnet's work and findings are not so open to the criticism which we have made of Adler's work. Nevertheless Burnet realized that this criticism might be made, and his answer to it has already been quoted. He thinks that if the criticism were valid, then similar findings of lesions and bacilli should have been made in other animals inoculated in a similar way, and not in only one out of six. It is a very great pity that Burnet did not attempt the crucial experiment of passage from the one animal to other animals. This is in our view the only method of proving an animal's susceptibility to human leprosy.

There is another possibility which although perhaps remote cannot be entirely ignored. May the hamster, like some other rodents either in the wild state or in captivity, barbour an infection caused by an acid-fast organism. As far as we know such an infection has not been reported in the hamster, but it may not have been looked for. If such an infection was present in Adler's or Burnet's animals and absent in ours, the divergence of findings might be explained.

Summary.

1. Attempts were made to infect twenty-three Syrian hamsters with human leprosy material, some of the animals being splenectomized and some not, some being inoculated intraperitoneally, and some subcutaneously by implantation of a small piece of nodule.

2. Six died in three weeks and two lived eight months, and the rest (15) were sacrificed between the ninth and twelfth months.

3. In nine out of thirteen animals in which fragments of nodule were implanted, the implants although not appreciably bigger than before, could still be found, and in these implants large numbers of acid-fast bacilli were found.

4. Slight macroscopic lesions and bacilli were found elsewhere than the nodule in only two of the twenty-three animals, and these lesions (in the form of slight enlargement and congestion of the local lymph nodes) were found in only two out of six animals dying early (3 weeks). No such lesions were found later.

A few bacilli but no visible lesions were found also in other sites in two other animals, once in the inguinal gland and once in the omentum.

These findings of bacilli were more frequently seen in animals examined early (3 out of 6) than in animals examined later (1 out of 17).

5. It is concluded that, although bacilli can persist in the implanted nodule for long periods, and although the possibility of their multiplication in such

implants has not been disproved, the inoculation of Syrian hamsters by this method or by intraperitoneal inoculation has not been followed by a generalized or progressive infection.

6. We have thus failed to confirm the reports of Adler and Burnet that the Syrian hamster is susceptible to human leprosy. Some possible explanations of this failure are discussed.

ACKNOWLEDGMENT.

Our thanks are due to Dr. R. O. A. Smith, Director, Pasteur Institute, Kasauli, for supplying hamsters and providing facilities for the experiment.

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N.B.—Since the above article was drafted we have seen a recent publication of Burnet (1939) in which he reports that a Syrian hamster fed on massive meals of leprous material and examined nine months later, showed numerous bacilli but no obvious lesions in lung, liver, spleen, kidneys and mesenteric glands. These findings are interpreted as confirming that the hamster is susceptible to human leprosy and showing that it can be infected by the oral route.

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A COMPARATIVE STUDY OF TWO STRAINS OF VACCINIA VIRUS.

BY

C. G. PANDIT, M.B., B.S., Ph.D., D.P.H., D.T.M.,

AND

R. SANJIVA RAO, M.B., B.S., D.T.M.

(From the King Institute, Guindy, Madras.)

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It is a common practice in most vaccine lymph institutes to maintain the potency of their ' seed ' lymph by periodical transfers on animals of different species, particularly rabbits. Whatever may be the origin of the vaccinia virus used, the strains have, without a doubt, passed through countless such serial passages. The question arises whether, on this account, any modifications in the antigenic characters of the virus have been brought about, particularly from the point of view of conferring protection against smallpox. Indeed, this view was expressed recently in a conference of the Madras Provincial Health Officers. At the suggestion of Lieut.-Colonel H. E. Shortt, I.M.S., the Director of the Institute, it was decided to investigate the problem by raising a new strain of vaccinia virus. It is now accepted by most workers that variola virus, by suitable passages on monkeys and calves, can be transformed into vaccinia virus. Such a strain was raised by Dr. S. R. Pandit at the Institute. On theoretical grounds such a newly raised strain would be the ideal one to employ in the routine manufacture of vaccine lymph. There is, however, no standard technique to determine whether such a strain is necessarily superior to the one in current use. Several authors, particularly Craigie (1932), Craigie and Wishart (1934), Parker and Rivers (1935) and Salaman (1937), have recently developed special methods to study the antigenic structure of vaccinia virus, employing for the purpose elementary body suspensions of the virus. In the present work an attempt has been made to ascertain by such methods the antigenic relationship between two strains of vaccinia virus—one in use in the routine manufacture

of calf lymph in this Institute, and the other developed from smallpox virus by the method indicated above. In the present paper, these are referred to as 'V' and 'S. P.' strains, respectively.

Material and methods.

It may be stated that the potency of this Institute strain has been maintained since 1922 by a modified Nijland cycle, i.e., passages in series through calves, rabbits, and buffaloes, the buffalo lymph being always used as seed lymph for vaccinating calves which yield the lymph for use in the field. The strain derived from smallpox virus has also been maintained by the same technique and was in its 43rd passage when it was used for these experiments.

Preparation of elementary bodies.

The method adopted for the preparation of elementary bodies was the same as that described by Craigie (*loc. cit.*) with the exception that calves were used instead of rabbits in the production of lymph. The technique of vaccinating calves was the same as is usually employed in the manufacture of vaccine lymph. About 25 to 30 grammes of crude lymph were thoroughly mixed with 90·0 c.c. of 0·004 M. citric acid disodium phosphate buffer, pH 7·1, and shaken well in a stoppered bottle for about 10 minutes. The mixture was then centrifugalized in an ordinary horizontal centrifuge for 5 minutes at 3,000 r.p.m. The supernatant fluid was set aside in a flask, but the sediment was thoroughly mixed with another 50 c.c. of the buffer solution and centrifugalized as before for 5 minutes. The second supernatant was added to the first and the sediment again mixed with 30 c.c. of the buffer solution and again centrifugalized as before for 5 minutes. This supernatant was also added to the previous pool. Usually three such extractions were sufficient to recover most of the elementary bodies from the material. The number of extractions, however, solely depends on the initial elementary body content of the crude lymph and has to be increased or decreased accordingly. This can be judged by the opacity of the supernatant at each extraction.

The pooled supernatant was then centrifugalized in an angle centrifuge at 3,500 r.p.m. for one hour. The supernatant which contained the specific soluble substance was stored in the refrigerator and the sediment, which contained practically all the elementary bodies, was suspended in a sufficient amount of buffer solution and centrifugalized in the angle centrifuge as before for one hour. This supernatant was discarded and the process of washing the elementary bodies was repeated two or three times. Finally, the deposit was re-suspended in the required amount of buffer solution and centrifugalized in the *horizontal centrifuge* at 3,000 r.p.m. for one hour. This final centrifugalization brings down practically all the cell-dèbris and bacteria. The supernatant, which contained all the elementary bodies, was pipetted off and stored under æther in the refrigerator. The whole process was conducted at room temperature (which varied between 80°F. and

90°F.) using chilled buffer solution throughout. This elementary body suspension was used for all the tests mentioned hereafter.

Preparation of hyperimmune sera.

Calves were used for this purpose. Large amounts of antisera were thus readily available and the possibility of any non-specific reactions resulting from the use of materials from animals of different species was also avoided.

Calves were first vaccinated in the usual way with the two strains of vaccinia virus, and were then given four intravenous injections of 10 c.c. each of a thick suspension of washed elementary bodies at weekly intervals. They were bled a week after the last injection. The serum was separated with aseptic precautions and stored in the refrigerator.

Cross-immunization tests in rabbits.

At the outset it was determined that the two strains of the virus conferred immunity against each other. Rabbits successfully vaccinated with the elementary bodies of one strain failed to react when re-vaccinated with the other strain three to four weeks later.

After primary vaccination each rabbit was bled, and the serum was used for virus neutralization tests using the elementary body suspensions of both the strains for the purpose. The following technique was adopted :—

Equal volumes of serial dilutions of sera and stock elementary body suspension of each virus strain were mixed together and allowed to stand for one hour at room temperature. Rabbits were vaccinated on the flank with skin scarification technique using 0·2 c.c. of each of the mixtures put up. The results were noted on the 3rd day. Table I gives the results obtained :—

TABLE I.

Virus neutralization tests with sera obtained from vaccinated rabbits.

Immune serum used.	Virus used in serum-virus mixture.	Serum dilution.			
		10	20	50	100
Serum (*vs.* S. P. strain).	'V' elementary bodies ..	—	—	±	+
	'S. P.' elementary bodies ..	—	—	±	±

± = Doubtful take. + = Good take.

TABLE I—*concld.*

Immune serum used.	Virus used in serum-virus mixture.	Serum dilution. 10	20	50	100
Serum (*vs.* V strain).	'V' elementary bodies ..	—	—	±	+
	'S. P.' elementary bodies ..	—	-	±	+

± = Doubtful take. + = Good take.

It will be seen that while the serum of rabbit vaccinated with S. P. strain neutralized the 'V' strain to a less extent than the homologous strain, the results on the whole do not suggest any antigenic differences between the two strains.

Determination of antigenic differences by cross-agglutination tests between the two strains of vaccinia virus.

Craigie and Wishart (*loc. cit.*) have shown that vaccinia virus consists of two serologically active components, viz., (*a*) elementary bodies and (*b*) specific soluble substance which is filtrable through Seitz EK discs. Each of these contains a heat-labile fraction inactivated by exposure to 56°C., or by treatment with 1 per cent formalin, and a heat-stable fraction, resistant to boiling and 1 per cent formalin, both the heat-labile and heat-stable fractions being antigenic and giving rise to specific antibodies.

In the further investigation of the antigenic relationship between the two strains, it was decided to conduct agglutination tests with the heat-stable fractions, in addition to the untreated elementary bodies and their corresponding antisera, with a view to investigate the antigenic similarity or otherwise between the two strains. The technique of the preparation of hyperimmune sera has already been described. In the following tests, hyperimmune sera 694 and 695 were prepared by injecting elementary bodies of 'V' and 'S P.' strains respectively, and sera 755 and 743 by injecting elementary bodies heated to 70°C. of 'V' and 'S. P.' strains respectively.

For agglutination tests the method adopted by Parker and Rivers (*loc. cit.*) was adopted. For dilution of the hyperimmune sera, 0·85 per cent saline containing 0·2 M. citric acid disodium phosphate buffer was used as this prevents spontaneous agglutination of the elementary bodies. For antigen stock suspension of elementary bodies was used, diluted with 0·004 M. citric acid disodium phosphate buffer to a suitable opacity which more or less corresponded with the opacity of bacterial suspensions used in agglutination tests. 0·25 c.c. each of the serum and antigen were used in ordinary Dreyer's tubes. The tubes were corked to prevent evaporation and incubated overnight in an incubator at 37°C. This gave better results than incubation in a water-bath. Normal calf serum was used as a control. The results are given in Tables II, III and IV :—

Table II.

Cross-agglutination tests with hyperimmune sera and elementary body suspensions of the two strains of vaccinia virus.

Immune serum.	Elementary bodies.	Serum dilution. 5	10	20	40	80	160	320
694 (*vs.* 'V' strain).	'V' strain ..	+++	+++	+++	++	+	±	–
	'S. P.' strain ..	++	+++	+++	++	–	–	–
695 (*vs.* 'S. P.' strain).	'V' strain ..	+++	+++	++	+	±	–	–
	'S. P.' strain ..	+++	+++	+++	++	+	–	–

+ , ++ , +++ = Degree of agglutination.

Table III.

Cross-agglutination tests with hyperimmune sera and heated elementary body suspensions of the two strains of vaccinia virus.

Immune serum.	Elementary body suspensions heated to 70°C.	Serum dilution. 5	10	20	40	80	160	320
694 (*vs.* 'V' strain).	'V' strain ..	+++	++	++	+	±	±	–
	'S. P.' strain ..	+++	++	+±	+	–	–	–
695 (*vs.* 'S. P.' strain).	'V' strain ..	+++	+++	++	+	–	–	–
	'S. P.' strain ..	+++	++	++	+±	+	–	–

+ , ++ , +++ = Degree of agglutination.

Table IV.

Cross-agglutinations with antisera against heat-stable fractions of elementary bodies and heated elementary body suspensions of the two strains of vaccinia virus.

Immune serum.	Elementary body suspensions heated to 70°C.	Serum dilution. 5	10	20	40	80	160	320
755 (*vs.* 'V' elementary bodies heated to 70°C.).	'V' strain ..	++	++	+	+	+	±	−
	'S. P.' strain ..	++	+±	+	+	±	±	−
743 (*vs.* 'S. P.' elementary bodies heated to 70°C.).	'V' strain ..	++	++	+	+	±	−	−
	'S. P.' strain ..	++	++	+	+	+	+	−

+, ++, +++ = Degree of agglutination.

It will be noticed from the above tables that there are no appreciable antigenic differences between the two strains 'V' and 'S. P.', which are revealed by the agglutination reaction. There are minor differences in the agglutinative titres between the homologous and heterologous antigens but they can hardly be accounted for by any distinct agglutinogenic differences between them.

Cross complement-fixation tests with the two strains of vaccinia virus.

For complement-fixation tests the usual methods were adopted using the respective elementary body suspensions as antigen. To 0·1 c.c. of each of undiluted, 1 in 2, and 1 in 5 dilutions of the hyperimmune sera inactivated at 56°C. for 10 minutes, was added 0·1 c.c. of the elementary body suspensions, diluted in accordance with the anti-complementary dose which was previously determined, and 0·1 c.c. of guinea-pig serum containing 3 m. h. d. of complement. The tubes were incubated in a water-bath at 37°C. for half an hour, and then 0·1 c.c. of 3 per cent sheep cells, sensitized with 5 m. h. d. of amboceptor, was added. The tubes were again incubated in the water-bath at 37°C. for half an hour when the results were read. The results are given in Table V :—

TABLE V.

Complement-fixation tests with homologous and heterologous antigens.

Immune serum.	Antigen.	Serum dilution.		
		Undiluted.	1—2.	1—5.
694 (*vs.* 'V' strain).	'V' strain ..	+ +	+ +	+
	'S. P.' strain ..	+	+	+
695 (*vs.* 'S. P.' strain).	'V' strain ..	+	+	–
	'S. P.' strain ..	+ +	+	+

+ / + + } -- Degree of fixation.

It will be seen that the results do suggest small differences between the two strains, but they are not significant enough to postulate any antigenic differences between the two strains. Unfortunately the sera did not show the antibody in higher dilutions, and so dilutions up to 1 to 5 only were put up.

ABSORPTION EXPERIMENTS.

Since the foregoing experiments failed to reveal any appreciable differences between the two strains studied, it was considered advisable to absorb the immune sera with the heterologous elementary body suspensions and to repeat the tests using homologous elementary body suspensions as antigens. For serum absorption, the technique employed by Salaman (*loc. cit.*) was adopted. The optimal cross-agglutination rate was first determined. For this purpose, to each volume of a serial twofold dilution of an elementary body suspension in 0·004 M. citric acid disodium phosphate buffer, pH 7·1, was added an equal volume of 1 in 20 dilution of serum in 0·2 M. citric acid disodium phosphate buffer saline, pH 7·1. After immediately shaking the tubes, small amounts of each mixture were drawn up in a capillary tube, the different dilutions being separated by air spaces. The capillary tube was sealed, kept at room temperature, and the different portions examined for agglutination with a hand lens. The optimal rates were indicated by the first agglutination occurring within 5 to 7 minutes. From a consideration of the dilution of the serum and the dilution of the elementary body suspension, the optimal absorbing dose of elementary bodies per c.c. was arrived at. The suspension of elementary bodies, containing the absorbing dose, was then centrifugalized in an angle centrifuge, and the deposited elementary bodies washed and mixed well with the proper amount of serum to be absorbed, and then incubated for one and a half hours at 37°C. The mixture was then spun in a multi-speed centrifuge at 18,000

r.p.m., and the clear serum pipetted off. The serum thus absorbed was put through the same process of absorption with another optimal dose of elementary bodies, and the process repeated till the elementary bodies used for absorption showed no visible sign of agglutination in the serum. It has been shown by Salaman (*loc. cit.*) that the absorption of serum is more efficient when done fractionally as above. The results of agglutination, complement-fixation and virus neutralization tests done in this way are given in Tables VI, VII and VIII :—

TABLE VI.

Agglutination tests with absorbed sera.

Immune serum.	Absorbed with elementary body suspension.	Elementary body suspension as antigen.	Serum dilution. 5	10	20	40	80	160	320
694 (*vs.* 'V' strain).	'S. P.' ..	'V'	++	+	±	-	-	--	—
	Unabsorbed	'V'	+++	+++	++	+±	+	+	—
695 (*vs.* 'S. P.' strain).	'V' ..	'S. P.'	+	+	±	—	—	—	—
	Unabsorbed	'S. P.'	+++	++±	++	++	±	—	—

+, ++, +++ = Degree of agglutination.

TABLE VII.

Complement-fixation tests with absorbed sera.

Immune serum.	Absorbed with.	Antigen.	Serum dilution. Undiluted.	1—2.	1—5.
694 (*vs.* 'V' strain).	'S. P. E. B.'	'V. E. B.'	++	+	±
	Unabsorbed ..	'V. E. B.'	++	++	+
695 (*vs.* 'S. P.' strain).	'V. E. B.' ..	'S. P. E. B.'	+	±	±
	Unabsorbed ..	'S. P. E. B.'	++	+	+

E. B. = Elementary body suspension.

+, ++ = Degree of fixation.

TABLE VIII.

Virus neutralization tests with absorbed sera.

Immune serum in 1—5 dilution.	Absorbed with.	E. B. in mixture.	Virus dilution. 1—10.	1—100.	1—500.	1—1000.
694 (*vs.* 'V' strain).	'S. P.' ..	'V'	++	+	—	—
	Unabsorbed ..	'V'	—	—	—	—
695 (*vs.* 'S. P.' strain).	'V. E. B.' ..	'S. P.'	+++	+++	++	++
	Unabsorbed ..	'S. P.'	++	+	+	—
Virus control ..	..	'V'	++	++	+	—
		'S. P.'	+++	+++	+++	+++

E. B. = Elementary body suspension.
+, ++, +++ — Degree of 'takes'.

It will be noticed from the above tables that the absorption of sera with the heterologous virus suspensions was not quite complete. The results, however, indicate that each strain of the virus had appreciably absorbed from the heterologous antiserum its own antibody. The results of complement-fixation tests are not very striking. In the virus neutralization tests, where the virus dilutions were employed instead of serum dilutions, the considerably less pronounced reactions with the 'S. P.' strain mixtures were accounted for by the fact that the elementary body content in the 'S. P.' strain of virus used in the mixtures was much greater than in the 'V' strain as is shown by the reactions with virus controls.

Discussion.

An analysis of the various results shows a close similarity in the antigenicity of the two strains of vaccinia virus studied. As has already been stated previously, the small differences noticed in the titres in the different cross tests were not such as could be accounted for by any appreciable qualitative or quantitative antigenic differences between the two strains. The agglutination tests with sera 743 and 755 showed an identity of the heat-stable fractions also in both the strains. Experiments conducted here have shown that the infectivity and complement binding antigens are bound up with the heat-labile fractions of the elementary bodies, and

the cross complement-fixation tests and virus neutralization tests reveal a close similarity between the heat-labile fractions of the two strains.

Downie (1939) investigated the relationship of the virus of natural cow-pox to vaccinia virus used in the routine production of calf lymph, and came to the conclusion that, while there was a close immunological relationship between the virus of spontaneous cow-pox and the virus of vaccinia in current use in laboratories, a closer analysis revealed the fact that the antigens, although similar, were not identical. The cow-pox strains he used, however, had been propagated only through 26 passages over a period of 10 months, and he admitted the possibility of a modification of the cow-pox virus towards a closer approximation with the 'standard' vaccinia virus.

In the present work the strain of vaccinia virus obtained from smallpox material had passed through 43 passages for a period of over 2 years, and as such its close approximation to the standard vaccinia virus was quite possible. This is confirmed by Downie, who, working with two strains of vaccinia virus, one of which was derived from smallpox, could not find any qualitative differences in the two strains.

Summary.

1. A comparative antigenic and immunological study of two strains of vaccinia virus, one, the routine strain used for vaccine lymph production and the other raised from smallpox material, has been made.

2. Protection tests on vaccinated rabbits and experiments with sera obtained from vaccinated rabbits suggest a very close similarity between the two strains.

3. Agglutination tests, complement-fixation tests, and virus neutralization tests with unabsorbed and absorbed hyperimmune sera confirmed the above findings.

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THE SUSCEPTIBILITY OF DOMESTIC FOWLS TO A STRAIN OF RABIES VIRUS OBTAINED FROM A JACKAL.

BY

N. VEERARAGHAVAN, M.B., B.S.,

AND

G. L. C. PHILIPSZ, D.T.M., I.M.D.

(*From the Pasteur Institute of Southern India, Coonoor.*)

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INTRODUCTION.

THE experiments to be described in this paper were undertaken in an attempt to ascertain whether a strain of rabies virus passaged through domestic fowls would develop more readily on the chorio-allantoic membrane of the developing hen's egg. As reported in a previous paper (Veeraraghavan and Philipsz, 1938), no evidence was obtained that serial passage among fowls facilitated the development of the virus on egg membrane.

It is generally believed that fowls are highly resistant to rabies infection. Von Lote (1904) reported the development of rabies in a cock 545 days after inoculation with a strain of 'street' virus obtained from a rabid dog. Lubinski and Prausnitz (1926) state that, even when rabies has developed in the bird, it is not easily transmissible to other birds and that, at best, it is capable of transmission for only a few serial passages.

In this paper a description is given of the serial passage among domestic fowls of a strain of rabies virus* obtained from a jackal. Local fowls were found to be readily susceptible to this strain of virus and it was possible to carry on serial passages in fowls without difficulty. After the 15th passage the virus became 'fixed'.

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* The possibility of a coincidental infection with an unknown neurotropic virus cannot be excluded with certainty although we have no reason to suspect the presence of such an unknown virus.

Materials and methods.

The strain of rabies virus used in these experiments was obtained from the brain of a jackal, suspected to be rabid, which was brought to the Institute for confirmation of the diagnosis. Microscopic examination of the brain of this animal revealed the presence of typical *Negri bodies*. A suspension of the rabid jackal's brain in normal saline solution was inoculated intracerebrally into a healthy domestic fowl. This bird developed rabies 29 days later, and the virus was subsequently passaged to another fowl by the intracerebral inoculation of a suspension of a small portion of brain cortex emulsified in normal saline solution. Many subsequent sub-passages were made to other fowls in series. At each serial passage in fowls a portion of the fowl brain suspension was inoculated intracerebrally into a control guinea-pig or rabbit. The diagnosis of rabies in all fowls, guinea-pigs, and rabbits, was confirmed by microscopical examination of the brain.

Serial passage of jackal virus among domestic fowls.

The results of serial passage of the virus obtained from the rabid jackal among domestic fowls are summarized in the Table (*see* end of paper). Fowl 1, which was inoculated direct from the jackal, developed symptoms of rabies 29 days after inoculation. The earliest signs were drowsiness, drooping of the head (*see* Plate II, fig. 1) and unsteadiness of the legs. Later, paralysis of the legs became apparent and this spread over the whole body so that the fowl was completely paralysed within three days (*see* Plate II, fig. 2). Death occurred four days after the onset of symptoms. Microscopic examination of the brain revealed the presence of typical *Negri bodies* measuring from 1μ to $4{\cdot}5\mu$ in diameter. No bodies with which these could be confused were detected in sections of the brains of normal fowls.

The strain of rabies virus originally isolated from the jackal and established in fowl 1 was subsequently subjected to 27 serial passages among fowls. As will be observed from the Table the incubation period was somewhat inconstant up to the 5th passage but from the 6th passage onwards the duration of the incubation period became progressively diminished until it became ' fixed ' at two days from the 15th passage onwards.

The duration of illness from the onset of symptoms till death was four days in fowl 1. This period was reduced to two days in the 2nd and 3rd passages and in all subsequent passages the duration of illness did not exceed 24 hours.

During the first six serial passages the signs observed in infected fowls were similar to those described above for fowl 1, although the predominant symptom varied somewhat in individual cases. From the 7th passage onwards the symptoms observed were practically constant. Drowsiness and drooping of the head could usually be detected within 48 hours after intracerebral inoculation and these signs were quickly followed by spasmodic contractions affecting chiefly the muscles of the neck. The latter produced neck movements resembling those which occur when

fowls are feeding but their lack of purpose and their persistence almost to the time of death were highly characteristic. Spasmodic contractions were intensified when the birds were disturbed or subjected to the influence of noise or bright light. Cyanosis and increased salivation were pronounced in many cases. The saliva was viscid. A sample of saliva collected from fowl 6 during the early stages of the disease did not prove to be infective to a guinea-pig into the neck muscles of which it was inoculated.

Microscopical examination of the brains of infected fowls during the earlier serial passages showed an abundance of typical *Negri bodies* (*see* Plate III, figs. 5 and 6), but in later passages the number and size of *Negri bodies* became progressively diminished. After 27 serial passages the presence of *Negri bodies* could be detected only after prolonged examination of serial sections and none exceeded 0·4μ in diameter. The morphological characters of the *Negri bodies* observed are referred to in greater detail below.

A suspension of the brain of the rabid jackal was introduced into one fowl by corneal scarification. Symptoms of rabies developed seven and a half months later and the fowl died within two days. The signs observed were similar to those described above. Microscopical examination of the brain revealed the presence of *Negri bodies*.

The susceptibility of animals and birds to rabies virus obtained from a jackal and passaged through fowls.

Guinea-pigs or rabbits were used as controls of the sub-passages among fowls of the strain of rabies virus obtained from the jackal. Up to the 3rd passage in fowls the control guinea-pigs died without showing characteristic symptoms of rabies, although sections of their brains showed typical *Negri bodies*. The guinea-pigs used as controls of the 4th to 11th passages in fowls showed an incubation period of from 6 to 15 days. After subsequent sub-passages among fowls, however, the incubation period in the control guinea-pigs did not exceed 3 days and the animals died on the third or fourth day after inoculation. *Negri bodies* were found in sections of the brains of guinea-pigs even when death occurred within 3 days of inoculation (*see* Plate III, fig. 8). The guinea-pigs used as controls of the 4th to 11th passages among fowls showed signs of *furious* rabies, but those used as controls of subsequent passages among fowls died of *dumb* rabies.

Rabbits showed signs similar to those observed in guinea-pigs when inoculated with the rabies virus passaged in fowls. One animal inoculated from fowl 21 (*vide* Table) became paralysed within 2 days of inoculation and died on the third day. *Negri bodies* were found in sections of its brain (*see* Plate III, fig. 7). Similar results were observed in rabbits inoculated with the virus from fowls in subsequent serial passages. Attempts were made to sub-passage the virus among rabbits using material obtained from a rabbit which had succumbed two and a half days after inoculation. This was successfully accomplished for three sub-passages but with each sub-passage the incubation period tended to increase.

Explanation of Plate II.

Fig. 1. Fowl showing drooping of the head.
,, 2. Fowl showing drooping of the head and paralysis of its legs.
,, 3. Fowl showing complete paralysis (about three hours before death).
,, 4. Pigeon showing complete paralysis (virus from fowl 22).
,, 5. Sparrow paralysed on the 6th day after inoculation (virus from fowl 24).

PLATE II.

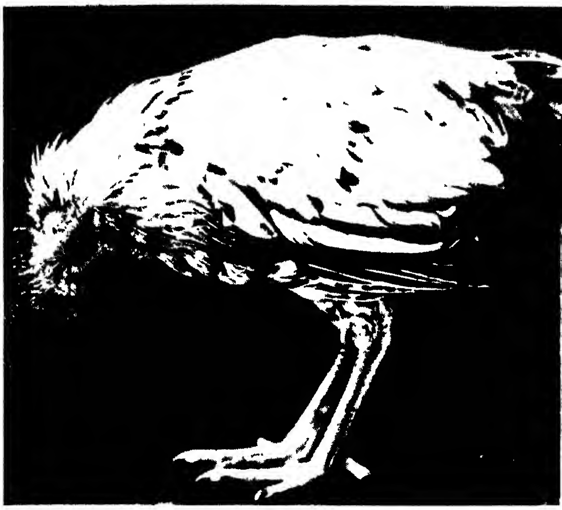

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Plate III.

Fig. 1.

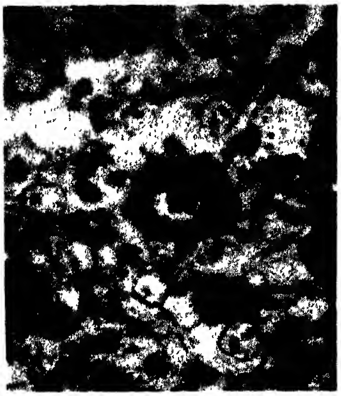

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

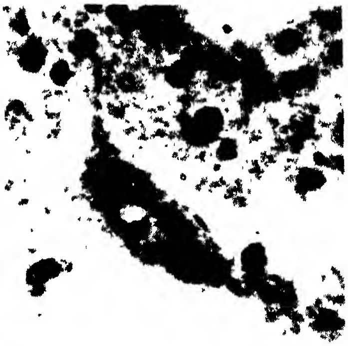

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9.

EXPLANATION OF PLATE III.

Fig. 1×900. Fragmented nucleolus of Purkinje cell of the cerebellum from fowl 10.

,, 2×900. Fragmented nucleolus of Purkinje cell of the cerebellum of fowl 10. A nucleolar fragment is apparently being extruded.

,, 3×900. Purkinje cell of the cerebellum of fowl 24 showing fragmented nucleolus and granules in the cytoplasm.

,, 4×900. Purkinje cell of the cerebellum of fowl 25 showing absence of nucleolus and numerous granules in the cytoplasm. All the granules were not apparent in the same focal plane.

,, 5×900. Typical *Negri body* in the cerebellum of fowl 1.

,, 6×900. Two typical *Negri bodies* in the cerebellum of fowl 16.

,, 7×900. Typical *Negri bodies* in the cerebellum of a rabbit which succumbed to jackal virus after 21 serial passages through fowls.

,, 8×900. *Negri body* in the cerebellum of a guinea-pig following infection with jackal virus after 18 passages through fowls.

,, 9×900. Section of the cerebellum of a pigeon showing presence of a *Negri body*. The pigeon was infected with jackal virus after 22 passages through fowls.

Pigeons inoculated intracerebrally with the jackal virus after passage through fowls developed rabies within 3 days, and died within 24 hours of the onset of symptoms. The symptoms resembled those observed in fowls. Paralysis commenced in the legs and spread rapidly ; the wings were involved early (*see* Plate II, fig. 4). Spasmodic contractions commenced early, occurring in rapid succession, and were highly characteristic. *Negri bodies* were observed in sections of the cerebellum and measured from $0{\cdot}3\mu$ to $0{\cdot}6\mu$ in diameter (*see* Plate III, fig. 9). Sub-passage of the virus obtained from the brain of a pigeon was effected without difficulty and, in contrast to the rabbit, there was no increase in the incubation period with serial sub-passage.

Sparrows inoculated with the virus obtained from fowls were relatively resistant to infection. The incubation period varied from 8 to 24 days and the duration of illness from 2 to 7 days. The chief symptom observed was paralysis which commenced in the legs and spread over the body. Sections of the cerebellum from infected sparrows showed *Negri bodies* measuring from $0{\cdot}4\mu$ to $0{\cdot}8\mu$ in diameter.

The susceptibility of fowls to Paris fixed virus.

Local domestic fowls were found to be highly resistant to the strain of Paris fixed virus employed at this Institute for the preparation of antirabic vaccine. Two fowls became infected 8 and 11 months respectively after inoculation which in one case was carried out intracerebrally and in the other by corneal scarification. *Negri bodies* measuring $0{\cdot}3\mu$ to $0{\cdot}8\mu$ in diameter were present in scanty numbers in the cerebellum of each of these two fowls.

Observations on the morphology of *Negri bodies*.

Examination of sections of the cerebellum of fowls infected with the strain of rabies virus obtained from the jackal showed, in the earlier passages, large numbers of *Negri bodies*. These measured from 1μ to $4{\cdot}5\mu$ in diameter and were morphologically typical (*see* Plate III, figs. 5 and 6). As the number of fowl to fowl passages increased the number and size of the *Negri bodies* tended to diminish. After 27 serial passages of the virus in fowls the number of the *Negri bodies* was so small that they could be found only after prolonged examination of serial sections. Those seen did not exceed $0{\cdot}4\mu$ in diameter.

After the 10th serial passage of the virus in fowls, sections of the cerebellum showed the presence of a number of Purkinje cells the cytoplasm of which presented a granular appearance. The number of granules present in a single cell was very variable. Sometimes only two or three round or oval granules were seen and sometimes large numbers of irregular granules scattered throughout the cytoplasm of the cell could be made out (*see* Plate III, fig. 4). These granules stained red with Mann's stain and took the iron stain when stained with iron-hæmatoxylin. As a rule the granules were observed in the cytoplasm of cells in which the nucleolus was not apparent (*see* Plate III, fig. 4) or in which the nucleolus was fragmented (*see* Plate III, figs. 2 and 3). Appearances which were consistent with the

extrusion of the nucleolus into the cytoplasm of the cell were sometimes observed (*see* Plate III, fig. 2). The occurrence of granules similar to those described above has been noted by Cornwall (1925) who referred to them as 'corpuscles'. He believed them to be a stage in the development of the 'parasite of rabies' following the breaking up of the *Negri body*. These granules or 'corpuscles' were not seen by us in the brains of fowls used for the earlier passages of the virus when typical large-sized *Negri bodies* were plentiful, and in subsequent passages they did not occur in sections in which typical *Negri bodies* were present in appreciable numbers. These observations together with the observed variability in the size and shape of the granules throw doubt on the hypothesis that they are stages in the development of the 'parasite of rabies'. The appearances simulating the extrusion of nucleoli into cytoplasm which we have observed are in accord with those described by Acton and Harvey (1911) who believed that *Negri bodies* are derived from extruded particles of nucleolar material. The finding of *Negri bodies* in cells in which the nucleus and nucleolus were apparently normal does not, however, support the contention of the latter workers.

Summary and conclusions.

A strain of rabies virus isolated from a jackal, infected in nature, has been found to be pathogenic for domestic fowls. In the original fowl passage the incubation period was 29 days but as the result of subsequent serial passages in fowls it was gradually reduced until, after the 15th passage, it remained 'fixed' at 2 days. At the same time the duration of illness in fowls was reduced from 4 days to 1 day. The most characteristic signs of the disease were increased salivation, drooping of the head, spasmodic contractions of the neck muscles, and paralysis commencing in the legs and later becoming general.

During the earlier passages of the virus among fowls, sections of the cerebellum showed numerous typical *Negri bodies* measuring from 1μ to $4{\cdot}5\mu$ in diameter. In later passages the size and number of recognizable *Negri bodies* became progressively diminished until, in the last few passages, they were present only in very scanty numbers and none exceeded $0{\cdot}4\mu$ in diameter.

The jackal virus 'fixed' by passage among fowls proved to be highly pathogenic for other animals. Rabbits and guinea-pigs showed symptoms from 2 to 3 days after intracerebral inoculation and died within 24 hours of the onset of detectable symptoms. Pigeons developed symptoms within 3 days of inoculation and died within 24 hours of the onset of symptoms. Sparrows were relatively resistant and showed not only a longer period of incubation but a more protracted period of illness.

Fowls inoculated with the strain of Paris fixed virus used at this Institute for the preparation of antirabic vaccine developed rabies only after a very protracted period of incubation.

A brief description is given of the appearance of granules in the cytoplasm of the Purkinje cells of the cerebellum. These granules varied in size, shape and number, and, when present, no typical *Negri bodies* could be detected in the same

sections. No support was found for the belief expressed by Cornwall (*loc. cit.*) that these ' corpuscles ' represent a stage in the development of the ' parasite of rabies '. The observed occurrence of granules in cells with apparently intact nuclei did not support the contention of Acton and Harvey (*loc. cit.*) that *Negri bodies* are derived from extruded nucleolar material. Appearances suggestive of the extrusion of nucleolar material from the nuclei of these cells were, however, occasionally observed.

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TABLE.

Serial passage among domestic fowls of a strain of rabies virus isolated from a naturally-infected jackal.

Serial passage in fowls.*	Source of virus.	Incubation period in days.	Duration of illness in days.	*Negri bodies.*		Animal used as control of fowl passage.	Incubation period in days.	Duration of illness in days.	*Negri bodies.*	
				Number.†	Size, μ.				Number.†	Size, μ.
Fowl 1	Jackal	29	4	+++	1 to 4·5	Guinea-pig	15	2	+++	0·5 to 11·5
,, 2	Fowl 1	34	2	+−+	0·5 to 2	,,	6	2	+++	0·5 to 10
,, 3	,, 2	21	2	+++	0·4 to 1·75	,,	5	2	+++	0·4 to 6·2
,, 4	,, 3	17	1	+++	0·4 to 1	,,	12	1	++	0·4 to 5·8
,, 5	,, 4	47	1	++	0·5 to 1·1	,,	12	2	++	0·4 to 4·2
,, 6	,, 5	6	1	++	0·4 to 0·9	,,	15	2	+	0·4 to 1·5
,, 7	,, 6	5	1	+	0·3 to 0·8	,,	9	2	+	0·5 to 1
,, 8	,, 7	3	>1	++	0·3 to 0·9	,,	5	2	+	0·4 to 0·8
,, 9	,, 8	4	1	+++	0·5 to 1	,,	5	2	++	0·4 to 1·8
,, 10	,, 9	4	1	+++	0·4 to 1	,,	7	3	+	0·4 to 1
,, 11	,, 10	2	1	+++	0·4 to 0·6	,,	5	3	+	0·4 to 1
,, 12	,, 11	3	1	−	0·4 to 0·8	,,	2	1	−	..
,, 13	,, 12	4	1	±	0·3 to 0·8	,,	2	1	−	..

* All passages were effected by the intracerebral inoculation 0·1 c.c. of a 5 per cent suspension of brain cortex in normal saline solution.

† +++ indicates that *Negri bodies* were prevalent.
++ ,, ,, ,, present in fair numbers.
+ ,, ,, ,, scanty.
± ,, ,, ,, detected only after prolonged examination of serial sections.
− ,, ,, ,, not found.

TABLE—*concld.*

Serial passage in fowls.*	Source of virus.	Incubation period in days.	Duration of illness in days.	*Negri bodies.* Number.†	*Negri bodies.* Size, μ	Animal used as control of fowl passage.	Incubation period in days.	Duration of illness in days.	*Negri bodies.* Number.†	*Negri bodies.* Size. μ.
Fowl 14	Fowl 13	5	1	+	0·4 to 0·8	..	..	..	..	..
,, 15	,, 14	2	>1	+	0·4 to 0·9	Guinea-pig	2	<1	—	..
,, 16	,, 15	3	<1	+	0·3 to 0·6	,,	2	1	+	0·4 to 0·8
,, 17	,, 16	2	<1	+	0·4 to 0·8	,,	2	1	++	0·4 to 1
,, 18	,, 17	2	1	+	0·3 to 0·7	,,	2	1	+	0·5 to 1·2
,, 19	,, 18	2	<1	±	0·4 to 0·8	,,	3	1	+	0·4 to 2
,, 20	,, 19	2	>1	±	0·3 to 0·5	,,	3	1	..	..
,, 21	,, 20	2	1	+	0·3 to 0·6	,,	2	2	+	0·4 to 1
,, 22	,, 21	2	1	±	0·3 to 0·5	Rabbit	2	1	+	0·4 to 2
,, 23	,, 22	2	>1	+	0·3 to 0·7	,,	2	1	—	..
,, 24	,, 23	2	>1	±	0·3 to 0·6	,,	2	1	+	0·4 to 1
,, 25	,, 24	2	1	±	0·3 to 0·5	Guinea-pig	2	2	—	..
,, 26	,, 25	2	1	±	0·3 to 0·4	,,	3	1	+	0·4 to 2
,, 27	,, 26	2	<1	±	0·3 to 0·4	..	..	..	..	..

* All passages were effected by the intracerebral inoculation 0·1 c.c. of a 5 per cent suspension of brain cortex in normal saline solution.

† ++ indicates that *Negri bodies* were present in fair numbers.

\+ ,, ,, ,, scanty.

± ,, ,, ,, detected only after prolonged examination of serial sections.

— ,, ,, ,, not found.

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THE NICOTINIC ACID CONTENT OF THE TISSUES OF MONKEYS FED ON WHEAT, MAIZE AND RICE DIETS.

BY

M. SWAMINATHAN.

(*Nutrition Research Laboratories, I. R. F. A., Coonoor, S. India.*)

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NICOTINIC ACID plays an important rôle in nutrition but little is known about its physiological action in the body. Data about the amounts contained in various tissues may throw light on this question. The present paper records an investigation of the nicotinic acid content of the liver, muscle and brain of monkeys.

Aykroyd and Swaminathan (1940) showed that comparison of the nicotinic acid content of poor rice and maize diets failed to throw light on the association between pellagra and maize. Though rice-eaters rarely develop pellagra, the nicotinic acid content of rice diets may be lower than that of maize diets consumed by groups in which the incidence of pellagra is high. Apparently a low dietary intake of nicotinic acid does not invariably give rise to pellagra. A possible explanation of the problem might be found in the fact that nicotinic acid is not well absorbed from maize diets. If the organs of animals fed on maize were found to contain less nicotinic acid than the organs of animals fed on rice, this might suggest that the nicotinic acid present in maize was not satisfactorily absorbed and assimilated. In the present investigation the nicotinic acid content of the liver, brain and muscle of monkeys fed on wheat, rice and maize diets, respectively, has been studied.

METHOD OF ESTIMATING NICOTINIC ACID.

A colorimetric method of estimating nicotinic acid in suitable aqueous extracts of foods and animal tissues, involving the use of cyanogen bromide and aniline, has been described in three previous communications (Swaminathan, 1938, 1938*a*, 1939). The principle of the method is based on the observation of König (1904) that a coloured compound is obtained when the pyridine nucleus reacts with cyanogen bromide and a primary or secondary amine. Since the cyanogen bromide

test for nicotinic acid is now being employed by workers in a number of laboratories, a brief account of some further observations which concern the method itself will not be out of place.

Various modifications in the colorimetric procedure have been proposed by certain other workers (von Euler, Schlenk, Heiwinkel and Hogberg, 1938; Bandier and Hald, 1939; Harris and Raymond, 1939). The test in any form requires the use of cyanogen bromide for the break-down of the pyridine ring, but in place of aniline these three groups of workers have used β-naphthylamine, metol and p-aminoacetophenone, respectively. They have also pointed out that the colour reaction is influenced by pH and the presence of certain inorganic salts.

The relative sensitivity of various methods.—The time required for the development of the maximum colour using the author's method with aniline is two minutes, while with von Euler's and Bandier's methods it is 120 and 60 minutes, respectively. The intensity of the maximum colour produced by standard amounts of nicotinic acid using the author's method was found to be twice and four times the intensity of the colour produced using Bandier's and von Euler's methods, respectively.

Influence of pH on the intensity of the colour reaction.—It was found that the maximum colour is produced when the pH is from 7 to 9, while the colour is less intense in the acid range (6 to 4·5). Hence it is essential that the pH of the standard and the unknown should be the same.

The effect on the colour reaction of varying the quantities of buffer.—Experiments in which varying amounts of phosphate buffer were added to a nicotinic acid solution, and reagents for the colorimetric test subsequently added, showed that variation in the quantity of buffer has no effect on the colour reaction.

Influence of inorganic ions on the colour reaction.—The influence of NaCl, Na_2SO_4, KNO_3, CH_3COONa and NH_4Cl at 10 per cent concentration on the colour reaction was studied. It was found that none of these salts influenced the intensity of the colour produced.

The estimation of nicotinic acid in animal tissues.

The method described previously (Swaminathan, 1938) can be used for estimating the nicotinic acid content of animal tissues. Preliminary work, however, showed that the lead acetate precipitation was unnecessary and that Norit charcoal could be used for decolorization of the extracts without reducing the yield. A simplified procedure was adopted as follows:—

Procedure.—A weighed amount (5 g. to 10 g.) of the finely-minced tissues was ground up with glass-powder and the mass transferred to a beaker (250 ml.) with about 100 ml. of distilled water. It was then heated in a boiling water-bath for 30 minutes with stirring, to convert reduced coenzymes into the oxidized form (von Euler *et al.*, *loc. cit.*). Fifteen ml. of concentrated hydrochloric acid (36 per cent) were then added and the heating was continued with stirring for another 10 minutes. The mixture was allowed to cool, centrifuged, and the clear centrifugate transferred into another beaker. The residue in the centrifuge tube was then washed with 50 ml. of 4 per cent hydrochloric acid and the washings added to the main bulk. The combined extracts (about 150 ml.) were concentrated to about 80 ml. by boiling in an open beaker. This resulted in the hydrolysis of the nicotinamide present into nicotinic acid. The solution was then cooled, just neutralized by the addition of caustic soda (50

per cent) and then made slightly alkaline (about N/10) by the addition of 0·5 ml. of caustic soda (50 per cent). Then 0·2 g. of Norit was added, and the mixture just brought to the boil and filtered while hot. The charcoal and filter-paper were washed with 30 ml. to 40 ml. of boiling water. The mixed filtrate, after cooling, was adjusted to pH 7, filtered, and made up to 150 ml., so that the concentration of nicotinic acid was 1·5 μg. to 3μg. per ml. depending upon the tissues under test. An aliquot (10 ml.) of the extract was used for the estimation of nicotinic acid. The solutions sometimes had a light yellow tinge, which was allowed for by a blank experiment.

Recovery of nicotinic acid added to tissues in the form of nicotinic acid and amide.—Known amounts of nicotinic acid and its amide were added to liver, muscle and brain, and the procedure described above was followed. The recovery was good in all cases. The results are given in Table I :—

TABLE I.

Recovery of added nicotinic acid and amide.

Tissue.	Amounts of nicotinic acid and nicotinamide added, mg.	Total nicotinic acid found, mg.	Recovery, per cent.
Liver, 5 g. ..	....	0·59	..
„ 5 g. ..	0·2 nicotinic acid 0·2 nicotinamide.	0·98	98
Muscle, 10 g. ..	....	0·58	..
„ 10 g. ..	0·1 nicotinic acid 0·2 nicotinamide.	0·85	90
Brain, 10 g. ..	....	0·46	..
„ 10 g. ..	0·2 nicotinic acid 0·2 nicotinamide.	0·82	90

Estimation of 'free' and 'bound' nicotinic acid in tissues.—In animal tissues nicotinic acid is found in 'free' and 'bound' form. So far as is known, the acid and the amide represent the 'free' form, while coenzymes I and II, which contain nicotinamide in their molecules, represent the 'bound' form. Von Euler *et al.* (*loc. cit.*) have described a method for the estimation of 'free' and 'bound' nicotinamide in tissues, based on the fact that the coenzymes are not soluble in 90 per cent alcohol, whereas nicotinic acid and its amide are fairly soluble. Green and Brosteaux (1936) found that acetone was as efficient as alcohol in precipitating coenzymes. A simple procedure was adopted as follows :—

Ten grammes of finely-minced tissue were placed in about 20 ml. of absolute alcohol or acetone and ground up with a little glass-powder. Eighty ml. of alcohol or acetone were added and the contents well stirred and filtered at the pump using a Buchner funnel. The residue in the funnel was washed with about 20 ml. to 30 ml. of alcohol or acetone. The combined filtrates, which contain the free nicotinic acid and the amide in solution, were used for the estimation of 'free' nicotinic acid.

'*Free*' *nicotinic acid.*—The combined filtrates were transferred quantitatively to a distilling flask, 2 ml. of 40 per cent sulphuric acid added and the solvent completely removed by distillation. The residue in the flask was washed down into a conical flask with 50 ml. of 7 per cent hydrochloric acid and boiled under reflux for half an hour. It was then allowed to cool, first neutralized and then made slightly alkaline (about N/10) using 50 per cent caustic soda. The alkaline solution was extracted once with about 30 ml. of ether to remove oily impurities, the ether in turn being washed once with 20 ml. of water and the washings added to the main bulk. After the addition of 0·2 g. of Norit, the mixture was just brought to the boil and filtered hot. The filter-paper and charcoal were washed with 20 ml. to 30 ml. of boiling water. The combined filtrates, after cooling, were adjusted to pH 7, concentrated, if necessary filtered, and made up to a convenient volume (100 ml. to 120 ml.), 10 ml. aliquots being used for the estimation of the nicotinic acid present.

'*Bound*' *nicotinic acid.*—The residue in the Buchner funnel was quantitatively transferred along with the filter-paper into a beaker by washing down with 100 ml. of distilled water. The procedure described above for the determination of 'total' nicotinic acid was followed.

The results of determinations of the total, 'free' and 'bound' nicotinic acid in the liver, muscle, and brain of two monkeys fed on wheat and rice diets, respectively, are shown in Table II :—

TABLE II.

Total 'free' and 'bound' nicotinic acid in animal tissues.

Mg./100 g.

Monkey number.	Diet.	Liver.			Muscle.			Brain.		
		Total.	Free.	Bound.	Total.	Free.	Bound.	Total.	Free.	Bound.
8	Wheat	13·9	8·0	5·3	5·4	3·8	1·7	..	..	..
30	Rice	11·3	5·8	5·2	5·7	3·0	2·8	1·2	1·9	2·2

The figures for free nicotinic acid in the tissues examined were high ; this was possibly due to post-mortem hydrolysis. The material was not available for chemical analysis until 2 to 3 hours after death.

THE NICOTINIC ACID CONTENT OF VARIOUS TISSUES OF MONKEYS FED ON DIETS BASED ON DIFFERENT CEREALS.

The composition of the diets fed to the monkeys is shown in Table III. These will be referred to as the wheat, maize, parboiled milled rice and raw milled rice diets, respectively. The wheat diet was a well-balanced one, containing, in addition to whole wheat, abundant quantities of milk products and vegetables. The rice diets resembled the usual diet of poor rice-eaters in India as determined by diet surveys. The rice diets were prepared and cooked according to the usual custom in South India, i.e., the rice was well washed before cooking and the cooking water discarded. It has been shown (Aykroyd and Swaminathan, *loc. cit.*) that this procedure reduces the nicotinic acid of rice by 40 to 50 per cent. The maize diet was similar to the rice diets except that whole yellow maize replaced rice and the maize was not washed before being cooked.

Approximate estimates of the daily intake of nicotinic acid are given in Table III. These are based on determinations of the nicotinic acid content of foods carried out in the laboratories, and, in addition, the nicotinic acid present in the rice diets was directly determined. The amounts present in quantities of the various diets yielding 2,600 calories, approximating to human daily intake, are also shown. This gives an idea of how much nicotinic acid a human being consuming diets of this nature would obtain. In all the diets the cereal supplied a large proportion of the nicotinic acid. The nicotinic acid content of cereals has been fully dealt with in a previous paper (Aykroyd and Swaminathan, *loc. cit.*) in which it was shown that parboiled milled rice contains more nicotinic acid than raw milled rice. This explains the difference in the nicotinic acid content of diets 3 and 4. The nicotinic acid content of whole maize lies between that of parboiled and raw milled rice, while whole wheat is considerably richer than rice or maize.

TABLE III.

Composition of diets.

1		2	3	4
Wheat diet.		Maize diet.	Parboiled milled rice diet.	Raw milled rice diet.
Whole-wheat flour 100·0 parts.		Whole-maize flour 100·0 parts.	Parboiled milled rice 100·0 parts.	Raw milled rice 100·0 parts.
Pulses 15·0 parts ..		Pulses 7·0 parts	..	..
Whole milk 150·0 parts ..		Vegetables 10·0 parts	..	..
Butter or ghee 10·0 parts		Gingelly oil 0·5 parts	The rest as in maize diet.	The rest as in maize diet.
Root vegetables 40·0 parts		Coco-nut oil 0·5 parts	..	..
Other vegetables 40·0 parts.		Common salt 0·5 parts	..	..
Fruits 20·0 parts ..		Chillies 0·25 parts	..	..
		Tamarind 0·25 parts	..	..
Approximate daily intake of nicotinic acid by monkeys (mg.).	4 to 5	1·2—1·5	1·6—2·0	0·6—0·8
Nicotinic acid per 2,600 calories (mg.).	31	11	16	6

Groups of monkeys were fed on these diets in the course of experiments carried out by other workers in the laboratories. The post-mortem material was obtained from 23 monkeys dying, or killed at the point of death, in the course of the experiments. All the animals whose tissues were tested had been on the experimental diets for at least 8 weeks; the majority had been under experiment for 4 to 6 months and a few for a longer period. Nearly all died of intestinal disease, after diarrhœa lasting 1 to 3 weeks. The tissues were usually obtained for examination 2 to 3 hours after death.

The nicotinic acid content of brain, muscle and liver was determined by the procedure described above. The results are shown in Table IV :—

TABLE IV

The nicotinic acid content of the tissues of monkeys fed on wheat, maize and rice diets.

Mg./100 g.

Diet.	Number of monkeys used.	Liver.	Muscle.	Brain.
Wheat diet ..	24	13·9	6·1	5·0
	8	13·9	5·4	..
	23	11·9	5·9	4·8
	21	11·2	5·4	4·9
	22	10·6	4·4	4·8
	39	10·7	5·7	4·8
	Mean	12·0	5·5	4·8
Maize diet ..	37	6·2	2·1	2·1
	38	11·8	4·4	3·4
	39	11·3	5·3	4·1
	40	7·4	3·1	3·8
	41	8·5	4·2	3·8
	Mean	9·0	3·8	3·4

The monkeys used were *Macacus sinicus*. Their weights lay between 2 kg. and 4 kg.

TABLE IV—*concld.*

Diet.	Number of monkeys used	Liver.	Muscle.	Brain.
Parboiled milled rice diet.	1	9·0	4·3	3·1
	2	8·3	4·8	4·5
	30	11·3	5·7	4·2
	36	10·2	5·1	4·4
	42	8·5	5·1	3·5
	60	9·2	5·2	..
	Mean	9·4	5·0	3·9
Raw milled rice diet.	33	6·7	3·8	2·4
	43	5·3	4·3	2·6
	46	7·2	4·0	4·6
	47	7·3	3·8	3·2
	61	7·0	4·0	3·2
	62	5·2	4·3	3·5
	55	7·0	3·3	..
	Mean	6·5	3·9	3·3

The monkeys used were *Macacus sinicus*. Their weights lay between 2 kg. and 4 kg.

The livers of the animals fed on the wheat diet had a higher average nicotinic acid content than the livers of the other groups. The lowest figures for liver were obtained in the raw rice group and throughout the groups the order of average values for the nicotinic acid content of liver corresponded with nicotinic acid intake. Differences in the case of muscle and brain were not very great, but the content of these tissues in the wheat and parboiled rice groups was higher than

in the other groups. In the raw rice group the values for liver, muscle, and brain were 55, 71 and 70 per cent, respectively, of those observed in the case of the group on the wheat diet.

It is important to note that the values for the tissues of the animals fed on maize were intermediate, being above those of the tissues of the raw rice group, and below those of the wheat group. No evidence was therefore obtained in support of the idea that the nicotinic acid present in maize is less 'available' than that contained in rice. These experiments have therefore failed to throw light on the problem of pellagra and its association with maize. It must be added that none of the monkeys fed on maize developed any of the characteristic signs of pellagra apart from diarrhœa.

In a previous investigation (Shourie and Swaminathan, 1939) it was found that the liver and muscle of rats fed on a diet resembling the wheat diet used in the present experiments had a nicotinic acid content of 15·2 mg. and 4·6 mg. per 100 g., respectively.

Kohn, Klein and Dann (1939) estimated the quantity of 'V-factor' or 'coenzyme-like substance' in the tissues of normal dogs and dogs suffering from black-tongue. They found that in black-tongue the amounts of V-factor present in muscle and liver respectively were 65 and 30 per cent of those found in normal animals. When the animals were cured by nicotinic acid, the percentages rose to 75 and 55, respectively, of the normal; this appeared to be compatible with normal health. Similar results have been reported by Axelrod, Madden and Elvehjem (1939). They found that in dogs with severe black-tongue the coenzyme 1 content of liver and muscle was about 60 per cent of that found in normal animals, while in dogs showing mild signs of black-tongue, the values for liver and muscle were 55 and 87 per cent of the normal, respectively. There is evidence that nicotinic acid is present in the tissues mostly in the form of coenzymes (von Euler *et al.*, *loc. cit.*). These results therefore support the finding that the nicotinic acid content of tissues is influenced by the nicotinic acid intake.

Summary.

1. The cyanogen bromide-aniline method has been adapted for estimating the nicotinic acid content of animal tissues.

2. Groups of monkeys were fed on diets based on whole wheat, whole maize, parboiled milled rice and raw milled rice, respectively. These diets contained approximately 31 mg., 11 mg., 16 mg. and 6 mg. of nicotinic acid per 2,600 calories, respectively.

3. The nicotinic acid content of the liver, muscle and brain of monkeys fed on the maize and rice diets was lower than that of the same tissues of monkeys fed on the wheat diet, the lower content of liver being particularly marked. The maize-fed group gave higher values than the group fed on raw milled rice.

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THE AVAILABILITY OF CALCIUM AND PHOSPHORUS IN CEREALS.

BY

K. V. GIRI.

(Nutrition Research Laboratories, I. R. F. A., Coonoor, S. India.)

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THE importance of ensuring an adequate intake of mineral elements is well known to nutrition workers. It has been shown by Aykroyd and Krishnan (1937) that poor rice diets are deficient in calcium. Milk, which is a good source of calcium, is scanty and costly in many parts of India. Cereals form the bulk of most Indian diets and the problem of how far cereals can supply essential mineral elements is of practical importance.

The question of the 'availability' of food constituents is receiving much attention at present. To be 'available' a food constituent must be absorbed and utilized in the body. The degree of availability is of importance in practical nutrition work since the intake of dietary constituents, as computed from tables of chemical composition, may be different from the quantity actually utilized in the body.

Fincke and Sherman (1935) have shown that the calcium of some foods may be rendered unavailable to the body in the presence of oxalic acid, which is present in foodstuffs, because of the loss of calcium as insoluble oxalate. Similarly, the phosphorus present in foodstuffs may be unavailable because it is present partly in organic combination as phytin. It has been shown by several workers (Bruce and Callow, 1934; McCance and Widdowson, 1935; Lowe and Steenbock, 1936) that phytin phosphorus is not completely available. In a previous communication (Giri, 1938) it was reported that cereals, nuts and legumes contain phytin phosphorus to the extent of 50 to 70 per cent of total phosphorus. The present investigation was undertaken with a view to determining, by metabolic experiments on rats, the availability of calcium and phosphorus present in cereals commonly used in South India.

Among the cereals, ragi contains a high concentration of calcium (0·334 per cent) as compared to rice (0·010 per cent), cholam (0·027 per cent) and cambu (0·049 per cent). It is therefore of interest to discover how far the calcium contained in ragi is available. Ranganathan (1935) has reported the percentage retention of calcium present in ragi, cambu, cholam and rice to be 42·6, 49·5, 70·4 and 87·1, respectively, while the corresponding values for phosphorus were 42·4, 54·1, 55·5 and 66·4.

Such studies are more likely to give clear-cut results when the mineral under investigation is the sole limiting factor in a diet complete with regard to all other components. It is, therefore, desirable to plan a diet which contains calcium and phosphorus in sub-optimal amounts, but is otherwise adequate. In the present investigation, the method adopted by Henry and Kon (1937) for the determination of the availability of calcium and phosphorus has been followed with slight modifications.

The cereals tested were rice, ragi (*Eleusine coracana*), cambu (*Pennisetum typhoideum*) and cholam (*Sorghum vulgare*). Since rice is very low in calcium, considerable experimental error would be involved in determining the availability of rice calcium, and no attempt was made to study it. The level of phosphorus intake may influence the utilization of calcium, so that the total phosphorus content of the test cereals is of importance. Since, however, ragi, cambu and cholam contain about the same amount of phosphorus, it was felt that the influence of this factor on calcium retention might be disregarded, and strictly comparable data with regard to calcium retention in the case of these cereals could be obtained.

Experiments were therefore undertaken :—

(*a*) To investigate the extent of the availability of calcium and phosphorus contained in cereals, when cereals constituted the main source of these elements. The phosphorus content of all the diets were almost equal, while calcium content varied.

(*b*) To determine the extent of the availability of calcium and phosphorus in ragi at different levels of intake, when the cereal constituted the main source of calcium and phosphorus.

EXPERIMENTAL.

Four groups of young albino rats weighing 60 g. to 70 g. and reared on the Coonoor stock diet, containing 4, 4, 4 and 6 animals respectively, were used. The rats were placed in metabolism cages so designed that the urine and fæces can be collected separately and quantitatively. In order to accustom the rats to the metabolism cages, a preliminary period of three days was allowed before the metabolism experiment commenced. The animals received the basal diet described below, which is deficient in calcium and phosphorus but contains other food factors in adequate amounts. The basal diet was supplemented with the cereal under investigation, which is the main source of phosphorus and calcium.

BASAL DIET LOW IN CALCIUM AND PHOSPHORUS.

	Parts.
Dried egg-white powder	14
Phosphorus-and-calcium-free salt mixture*	3
Sugar	10
Coco-nut oil	5

Seventy-five parts of the test cereal in powder form were included in the basal diet, the cereal forming about 70 per cent of the total ration. The test cereal was the main source of calcium and phosphorus.

The diet was supplemented by two drops of cod-liver oil daily, to supply vitamins A and D. The rats also received 20 mg. of an acid clay adsorbate of vitamin B_1 daily.

The four groups were as follows :—

Group	I	basal diet	+ ragi.
,,	II	,,	+ cambu.
,,	III	,,	+ cholam.
,,	IV	,,	+ rice.

The phosphorus and calcium contents of these diets are shown in Table I :—

TABLE I.

Total phosphorus and calcium content of the experimental diets.

Diet number.	Main source of P and Ca.	Phosphorus, per cent.	Calcium, per cent.	Ca/P.
I.	Ragi ..	0·175	0·240	1·37
II.	Cambu ..	0·227	0·080	0·35
III.	Cholam ..	0·182	0·058	0·32
IV.	Polished rice ..	0·102	0·010	0·10

	Parts.
*Sodium chloride	44·0
Potassium citrate	250·0
Magnesium sulphate (hydrated)	60·0
Iron citrate	10·0
' Trace mixture '	1·45

'TRACE MIXTURE'.

	Parts.
Potassium iodide	12
Sodium fluoride	10
Manganese sulphate (anhydrous)	2

TABLE II.

Intake, excretion and balance of phosphorus in rats receiving diets in which cereals constituted the main source of P.

Period three weeks.

Diet.	Rat number.	Total phosphorus intake, mg.	PHOSPHORUS EXCRETION, MG.				Phosphorus balance, mg.	Phosphorus retention, per cent.	Average phosphorus retention, per cent.
			Urine.	FÆCES.		Total.			
				Total P.	Inorganic P.				
I. Ragi ..	1	383	4·6	146	41	151	232	60·5	58
	2	362	6·2	120	36	126	236	65·1	
	3	389	4·2	194	70	198	191	49·1	
	4	390	6·0	167	43	173	217	55·7	
II. Cambu ..	5	419	18·0	109	37	127	292	69·7	74
	6	384	10·7	66	25	77	308	79·9	
	7	444	19·3	97	34	116	328	73·8	
	8	361	12·1	89	35	101	260	72·0	
III. Cholam	9	320	12·0	90	36	102	219	68·3	67
	10	328	7·0	99	35	106	222	67·8	
	11	314	13·0	105	50	118	196	62·3	
	12	306	7·6	85	29	93	213	71·0	
IV. Rice ..	13	155	11·0	50	30	61	94	60·8	64
	14	168	6·5	44	22	51	117	70·0	
	15	144	6·4	49	24	55	89	61·7	
	16	136	4·3	49	27	53	83	64·1	
	17	128	7·1	50	29	57	71	60·9	
	18	123	8·8	43	27	52	71	65·0	

TABLE III.

Intake, excretion and balance of calcium in rats receiving diets in which cereals constituted the main source of Ca.

Period three weeks.

Diet.	Rat number.	Total calcium intake, mg.	Calcium excretion, mg.			Calcium balance, mg.	Calcium retention, per cent.	Average calcium retention, per cent.
			Urine.	Fæces.	Total.			
I. Ragi ..	1	525	11·0	137	148	377	71·8	68
	2	496	5·7	109	115	381	76·7	
	3	533	6·7	205	212	321	60·2	
	4	535	5·7	187	198	337	63·0	
II. Cambu ..	5	148	3·8	18	21	127	85·7	90
	6	136	3·5	10	14	122	90·0	
	7	157	3·9	13	17	140	90·0	
	8	128	2·0	12	14	114	90·0	
III. Cholam ..	9	104	4·6	13	18	86	83·0	84
	10	105	1·0	17	18	87	82·4	
	11	100	3·0	12	15	85	85·0	
	12	98	1·5	11·5	13	84	86·6	

The quantity of food offered was always in excess of the daily intake. The food intake for each rat was calculated by deducting the residue left each morning from the quantity given on the previous day. Each experiment lasted for three weeks. Phosphorus and calcium balance was determined by measuring the total intake of these elements and the quantity excreted in the urine and fæces.

For the analyses, the fæcal material was ashed in platinum dishes. The ash was dissolved in dilute HCl and made up to a known volume. Calcium was estimated by the usual method of precipitating the calcium as calcium oxalate, and titrating with potassium permanganate. For the estimation of calcium in urine, a similar procedure was adopted. The urine was evaporated to dryness on a water-bath ; the semi-dried material was then ashed in platinum dishes.

Total phosphorus in urine and fæces was determined by the method of King (1932), and inorganic phosphorus by that of Fiske and Subbarow (1925).

Intake, excretion and balance of phosphorus and calcium in the various groups are shown in Tables II and III.

TABLE IV.

Average increase in weight of rats.

Diet.	Group.	Initial weight, g.	8 days, g.	14 days, g.	22 days, g.
Ragi ..	I	68·0	86·5	95·5	106·0
Cambu ..	II	66·0	74·0	80·0	88·5
Cholam ..	III	65·5	77·0	83·0	95·0
			4 days.	12 days.	22 days.
Rice ..	IV	62·5	68·0	74·0	78·0

The availability of calcium and phosphorus at different levels of intake when ragi constituted the main source of these minerals.

The investigations on the availability of calcium, when the test cereal constituted about 70 per cent of the diet, showed that the percentage retention of ragi calcium was lower than that of calcium in other cereals. Since ragi has a high calcium content, it was thought possible that the calcium intake on the ragi diet might have been above the normal requirement for the rat. Accordingly, the retention of ragi calcium at lower sub-optimal levels of intake was studied. The method of investigation was the same as described above, except that the proportion of the cereal in the diet was reduced to about 40 and 20 per cent in two experiments respectively, by replacing it by equivalent portions of starch. The results of these experiments are set out in Tables V and VI, which show

Table V.

The availability of Ca from ragi at different levels of calcium intake.

Total Ca content of the diet, per cent.	Ratio Ca/P in the diet.	Rat number.	Total intake, mg.	Ca excretion, mg.			Ca balance, mg.	Ca retention, per cent.	Average Ca retention, per cent.	Increase in weight of rats.	Average increase in weight.	Ca retention mg. per g. gain in weight.
				Urine.	Fæces.	Total.						
0·240	1·31	1	525	11·0	137	148	377	71·8	67·9	44	38	8·6
		2	496	5·7	109	115	381	76·7		33		11·5
		3	533	6·7	205	212	321	60·2		44		7·3
		4	535	5·7	187	198	337	63·0		33		10·2
0·140	1·24	1a	290	10·0	21·3	31·3	259	89·2	87·2	42	37·8	6·2
		2a	281	12·2	19·5	31·7	249	88·7		35		7·1
		3a	287	14·6	22·7	37·3	250	87·0		38		6·6
		4a	271	20·5	23·0	43·5	228	83·9		36		6·3
0·072	1·13	5a	135	15·8	13·4	29·2	106	78·4	84·1	25	22	4·2
		* 7a	123	4·8	9·6	14·4	109	88·3		21		5·2
		8a	132	6·0	13·1	19·1	113	85·5		20		5·6

* Rat No. 6a died before the experimental period.

Table VI.

The availability of P from ragi at different levels of P intake.

Total P content of the diet.	Ratio Ca/P in the diet.	Rat number.	Total P intake, mg.	P excretion. Urine.	P excretion. Fæces. Total P.	P excretion. Fæces. Inorganic P.	P excretion. Total.	P balance, mg.	P retention, per cent.	Average P retention, per cent.	Increase in weight of rats.	Average increase in weight.	P retention mg. per g. gain in weight.
0·175	1·31	1	383	4·6	146·6	41·0	151	232	60·5	57·6	44	38	5·3
		2	362	6·2	120·2	36·0	126	236	65·1		32		7·4
		3	389	4·2	194·0	70·3	198	191	49·1		44		4·6
		4	390	6·0	167·0	42·5	173	217	55·7		33		6·6
0·113	1·24	1a	235	7·4	28·2	10·1	35·6	199	84·7	79·1	42	37·8	4·7
		2a	226	3·1	45·9	11·2	49·0	177	78·3		35		5·0
		3a	231	5·3	46·8	13·2	52·1	179	77·5		38		4·7
		4a	218	3·4	48·1	16·1	51·6	166	76·1		36		4·6
0·064	1·13	5a	120	5·8	31·2	9·8	37·0	83	69·2	70·4	25	22	3·3
		* 7a	110	4·7	27·0	9·0	31·7	78	71·0		21		3·7
		8a	117	3·1	30·7	7·8	33·8	83	71·0		20		4·1

* Rat No. 6a died before the experimental period.

the excretion, intake and balance of calcium and phosphorus respectively in the various diets. The increase in weight of the rats is also shown. It was found that with smaller intakes of ragi calcium and phosphorus there was an increase in percentage retention.

Discussion of results.

The data presented above show that when the test cereal constituted 70 per cent of the diet, the phosphorus contained in the various cereals is not completely available for the rat. The low figures obtained for the availability of phosphorus may be due to the fact that a large part of the phosphorus present in cereals exists as phytin which has been shown to be a poorly available source of phosphorus for the rat (Lowe and Steenbock, *loc. cit.*). The percentage of available phosphorus in ragi, cambu, cholam and rice, reckoned as non-phytin phosphorus, is 30, 51, 40 and 59, respectively (Giri, *loc. cit.*). The corresponding values obtained in the present study by balance experiments were, however, 58, 74, 67 and 64. It is clear, therefore, that phytin phosphorus is not completely unavailable. Patwardhan (1937) recently showed that an enzyme capable of hydrolysing phytin is present in the intestine of the albino rat. This enzyme may bring about the partial conversion of phytin phosphorus into the available non-phytin phosphorus in the intestines.

The percentage retention of ragi calcium (67·9 per cent) was found to be lower than that observed in the case of cambu (89·0 per cent) and cholam (84 per cent). The availability of calcium may however vary with the level of calcium intake. Sherman and MacLeod (1925) have shown that rats increasing in weight from 45 g. to 200 g., deposit calcium at a rate slightly less than 10 mg. per gramme of gain in weight. The intake of calcium per gramme increase in weight of rats fed on the ragi diet was about 12 mg. to 16 mg., while the corresponding values for cambu and cholam were 4·0 mg. to 8·5 mg. and 3·0 mg. to 3·6 mg., respectively. The intake of calcium from the ragi diet was thus above the amount needed for the optimal retention of calcium during growth.

Further experiments in which intake of ragi was varied showed that the percentage retention of ragi calcium increases from 68 at 0·240 per cent calcium level to about 87 and 84 at 0·140 and 0·072 per cent calcium levels, respectively (Table V). When the mineral is present in the diet in sub-optimal amounts, the availability of ragi calcium is therefore as high as that of cholam and cambu calcium.

All the animals excreted phosphorus and calcium chiefly in the fæces at different intake levels.

As in the case of calcium, the retention of phosphorus contained in ragi is also different at different levels of intake (Table VI). Thus, the availability on a diet containing 0·175 per cent phosphorus was 58 per cent, while the corresponding figures with lower levels of phosphorus intake (0·113 and 0·084 per cent phosphorus) were 79 and 70, respectively. The lower figure obtained for the availability of

phosphorus in the diet containing 0·175 per cent phosphorus is probably due to its occurrence in the diet in excess of the amount required. In the case of rats receiving diets containing 0·113 and 0·04 per cent phosphorus, the mineral is present in sub-optimal amounts. In the ragi diets, the calcium/phosphorus ratio was virtually the same at all levels of ragi intake, and its possible influence on retention may therefore be disregarded.

It is clear that ragi constitutes the best source of calcium and phosphorus among the cereals investigated. Not only it is rich in calcium and phosphorus, but the percentage retention of these minerals, particularly calcium, is high. It is said that during rapid growth and calcification the calcium/phosphorus ratio of the diet should be somewhere between 1 and 2. A distorted calcium phosphorus ratio has been found to produce rickets in experimental animals. The calcium/phosphorus ratio in the diet containing 70 per cent of ragi was of the order of 1·31, while in the other cereal diets based on cambu, cholam and polished rice the values were 0·35, 0·32 and 0·10, respectively. It is clear, therefore, that the calcium/phosphorus ratio in ragi is very favourable for optimal growth and retention of these minerals. The calcium/phosphorus ratio in ragi is of the same order as the ratio in milk, i.e., 1·3 to 1.

Of the diets containing 70 per cent of the various cereals, the ragi diet produced the greatest increase in weight (Table IV). It cannot, however, be assumed that this effect was due to the higher intake of calcium alone, since differences may exist between the cereals as regards protein and other necessary food factors.

Summary.

1. The availability of calcium and phosphorus in rice, ragi (*Eleusine coracana*), cambu (*Pennisetum typhoideum*) and cholam (*Sorghum vulgare*) has been determined by balance experiments on rats.

2. When the test cereal constituted the main source of calcium and phosphorus in diets containing about 70 per cent of the cereal, and complete with regard to all other components, the values obtained for the availability of calcium and phosphorus were as follows :—

Calcium : Ragi, 68, cambu, 89 and cholam, 84.

Phosphorus : Ragi, 58, cambu, 74, cholam, 67 and rice, 64.

3. When the percentage of ragi in the experimental diet was reduced to about 40 and 20, so that calcium was supplied in amounts below that quantity necessary for optimum retention during growth, the values obtained for the availability of calcium in ragi were 88 and 84 per cent. The corresponding values for phosphorus were 79 and 70 per cent.

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A NOTE ON URINARY PORPHYRIN EXCRETION IN CASES OF STOMATITIS OF DIETETIC ORIGIN.

BY

R. PASSMORE,

T. SOMMERVILLE,

AND

M. SWAMINATHAN.

(*Nutrition Research Laboratories, I. R. F. A., Coonoor, S. India.*)

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THE pigment coproporphyrin I has been shown to be a normal constituent of urine. The figures for daily excretion vary from 30 to 50 micrograms (Rimington and Goldblatt, 1940) to 60 to 120 micrograms (Dobriner, Strain and Localio, 1937). Increased excretion has been found to occur in certain diseases, notably conditions associated with impaired liver function or increased hæmopoietic activity. The discovery of the success of nicotinic acid therapy in pellagra has resulted in an intensive study of the disease, especially in America. It has been claimed (Beckh, Ellinger and Spies, 1937 ; Spies, Cooper and Blankenhorn, 1938 ; Spies, Bean and Stone, 1938) that an increased excretion of urinary porphyrins is a regular feature of the condition and that this disappears *pari passu* with the signs and symptoms on treatment.

The method of assay of urinary porphyrins used by Beckh, Ellinger and Spies depended upon the estimation of the total pigments removed by 25 per cent hydrochloric acid from ether extracts of urine. It is open to the criticism that other pigments besides porphyrins are removed from ether extracts by this concentration of hydrochloric acid. Watson (1939) has demonstrated that a pigment urorosein is partially responsible for the colour of the 25 per cent hydrochloric acid extract. By the use of less concentrated hydrochloric acid it is possible to remove porphyrins from ether extracts with only occasional traces of other pigments. Dobriner *et al.* (1938), who used 0·5 per cent hydrochloric acid for extracting the porphyrin

pigment from ether, found a daily excretion of 240μg. of porphyrin in the urine in a case of alcoholic pellagra. This amount fell to within normal limits on treatment with liver extract and yeast. But these authors did not find that pathological porphyrinuria was an invariable accompaniment of this disease.

The stomatitis of dietetic origin common in South India has been shown to be due to deficiency of the vitamin-B_2 complex, but the actual factors in the complex which are concerned are a matter of dispute (Aykroyd and Krishnan, 1936). Katzenellenbogen (1939) has described cases of endemic glossitis in Palestine, in which the raw and fissured condition of the tongue resembled closely that found in South India and reacted favourably to nicotinic acid. In apparently similar cases in Singapore, Landor (1939) did not observe any improvement with the same treatment. In South India, Aykroyd, Krishnan and Passmore (1939) found that nicotinic acid had a curative effect on some cases, while others failed to benefit. In the circumstances we felt that a steady porphyrin excretion by stomatitis cases was worth undertaking and might throw some light on the relation between stomatitis, nicotinic acid deficiency and pellagra. The excretion by 5 cases before and after administration of nicotinic acid has been determined.

METHOD.

For the determination of porphyrin we followed a method, the details of which were kindly supplied by Dr. C. Rimington of the National Institute of Medical Research, Hampstead, London, to whom we are also grateful for a sample of pure coproporphyrin I as a standard.

Twenty-four-hour samples of urine were collected. One-tenth of the volume of glacial acetic acid was added and the whole extracted twice with half the volume of peroxide-free ether for 10 minutes each time. Any emulsion formed was removed by the addition of a little more acetic acid. The ether extract was then washed 4 to 5 times with glass-distilled water. Finally, 5 per cent by weight hydrochloric acid, in quantities of 1 ml. to 2 ml. at a time, were added and the mixture shaken vigorously. Three to five shakings were sufficient to extract all the porphyrins. The extraction was continued until no further purple colour was obtained. The volume was then made up to 10 ml. The extracts were usually of the pure purple colour of the standard porphyrin solution and showed the characteristic absorption bands. Sometimes traces of urobilin were also present, which could easily be differentiated spectroscopically. The amount of porphyrin present was determined by comparing the intensity of the characteristic absorption bands with that of standard solutions.

RESULTS.

Five typical cases of stomatitis were investigated. These were all living on a diet composed largely of parboiled rice with a limited amount of vegetables, meat and milk. The nicotinic acid content of such diets has been estimated to be about 10 mg. per day (Aykroyd and Swaminathan, 1940), which is low compared with that of good mixed diets.

Twenty-four-hour samples of urine were collected for 3 days prior to treatment and for 3 days following the commencement of treatment with nicotinic acid (100 mg. to 400 mg. daily). In each case definite improvement in the clinical condition occurred. No obvious change occurred after the administration of nicotinic acid. Thirty-two samples of urine from these cases were subsequently examined for porphyrin and in no instance was the urinary excretion of porphyrin found to be more than 50 micrograms per day. The 32 samples were pooled and the porphyrin re-extracted. In all, 600 micrograms of porphyrin was obtained, indicating an average daily excretion of about 20 micrograms.

Summary.

The urinary porphyrin excretion of persons living on a poor rice diet with a low nicotinic acid intake, and showing signs of stomatitis and glossitis attributable to a defective intake of the vitamin-B_2 complex, was below 50 micrograms per day, i.e., within normal limits.

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INVESTIGATIONS INTO THE DIETARY AND PHYSIQUE OF ABORIGINALS IN SANTAL PARGANAS, A DISTRICT OF BIHAR.

BY

K. MITRA.

(Nutrition Scheme, Public Health Laboratories, Bankipur, Patna.)

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INTRODUCTION.

General description.—The district of Santal Parganas situated at the south-eastern portion of the province of Bihar lies between 23° 40′ and 25° 18′ north latitude and between 86° 28′ and 87° 57′ east longitude and covers an area of about 5,470 square miles. According to the last (1931) census report the district included a population of 1,794,908 persons of which about seventy per cent were returned as aboriginals or semi-aboriginals.

Physical aspects.—Excluding a strip of low alluvial land at its north and eastern portion covering an area of about 500 square miles the district can be divided into a 'hilly portion' and 'undulating country'. The hilly backbone roughly runs north and south, made up of a medley of hill ranges and valleys and includes the whole of *Damin-i-koh* (literally skirts of hills) or *damin* tract as it is commonly known; this is a Government state covering an area of about 1,500 square miles. The hilly portion is covered by jungles (reserved forests) and very sparsely populated by the *Pakaria* (aboriginal) tribes. In the neighbourhood of these villages, which are few and far between, *korao* or primitive type of cultivation has been allowed by the government and the hills have been partly denuded of the jungle. The majority of the villages are situated in the valleys and surrounded by 'cultivated clearings'. The 'undulating country' extends over the west and the south-west of the district and consists of long ridges with intervening depressions, at some places rocky and covered with scrub jungle.

Public health.—Certain portions of the 'undulating country' have the reputation of being a health resort and consequently attract a large number of visitors from the neighbouring province of Bengal from the middle of September to the middle of February. In certain areas of the 'hilly portion' (including the damin tract) of the district with the defective drainage, water stagnates and people

suffer from malaria. The other major diseases prevalent are cholera and smallpox during epidemic seasons. Leprosy is also prevalent in the district to a moderate degree. The birth rate per mille, death rate per mille and deaths under one year per mille live births for the district as also for the whole province are given in Table I. It is admitted that there is a certain amount of under-reporting in connection with such figures but for purposes of comparative study these indices have their uses with the usual limitations.

TABLE I.

Vital indices of Bihar and Santal Parganas.*

Locality.	Birth rate per mille population.			Death rate per mille population.			Deaths under one year per mille live births.		
	1936	1937	1938	1936	1937	1938	1936	1937	1938
Santal Parganas ..	25·5	27·8	26·3	18·2	16·7	18·4	78	70	79
Bihar Province ..	35·5	34·3	34·5	21·7	22·5	23·6	118	116	120

* Compiled from the *Annual Public Health Reports.*

POPULATION, FOOD HABITS AND AGRICULTURE.

People.—The principal aboriginals in the district are: (1) Santals, (2) Sauria-paharias and (3) Malpaharias. The percentages of their numerical strength in relation to the total population in the district are 42·1, 3·4 and 2·1, respectively. Thus, it appears that the Santals are the predominant race in the district. The Paharias are mainly found in the Damin-i-koh, Saurias in the north and Mals in the south, and live on hill sides and tops, whereas the richer valleys are inhabited by the more enterprising Santals. The Paharia villages consist of collections of 15 or 20 huts; villages containing 40 or 50 huts can occasionally be seen, but are very rare.

The huts of the Paharias are made of bamboo and other wild grass and reeds and thatched with straw. In spite of being dark inside they allow of plenty of ventilation. The walls of the Santali huts are made of mud, neatly plastered with a mixture of cowdung and earth, no windows being provided. The thatches consist of straw only. Kitchen gardens are occasionally attached to the Paharia huts. These are a common feature around the huts of the Santals.

In contrast to the Paharias, the Santals appear to be more industrious and cleanly in their habits. Though all these aboriginals have taken to agriculture in varying degrees, yet none of them raise either cattle, goats or sheep. Without

exception every family possesses a flock of poultry (country fowl) and the majority of Santals maintain piggeries in addition.

During the period of investigations 491 adult (over the age of 18) Santals and 207 adult Paharias were weighed and measured. The average measurements of height in inches and weight in pounds in each of the groups with their standard deviations and standard errors are shown in Table II. These figures give some idea as to the stature and build of the adult population under reference. On statistically analysing the difference it appears that the difference in heights and weights observed between the sexes in each class is *real*. Further analysis reveals the fact that adult Santal males are *significantly* taller and heavier than either class of adult Paharia males. This is found to be true in the case of female sex also. But when the two classes of Paharia adults are compared to each other in their respective sex groups the differences noticed in stature and weight are found to be '*not significant*'.

TABLE II.

Average heights and weights of aboriginal adults in Santal Parganas.

Classes of aboriginals.	Sex.	Number examined.	Stature in inches. Mean.	Stature in inches. Standard deviation.	Weight in pounds. Mean.	Weight in pounds. Standard deviation.
Santals	Male	313	62·71 ± 0·13 S. E.	2·26 ± 0·09 S. E.	99·03 ± 0·65 S. E.	11·49 ± 0·45 S. E.
	Female	178	58·39 ± 0·20 S. E.	2·65 ± 0·14 S. E.	87·02 ± 0·85 S. E.	11·33 ± 0·60 S. E.
Malpaharias	Male	60	61·54 ± 0·26 S. E.	2·04 ± 0 19 S. E.	91·42 ± 1·07 S. E.	8·32 ± 0·76 S. E.
	Female	43	57·73 ± 0·25 S. E.	1 62 ± 0·17 S. E.	79·24 ± 1·3 S. E.	8·88 ± 0·96 S. E.
Sauriapaharias	Male	69	61·51 ± 0·23 S. E.	1·91 ± 0·16 S. E.	90·98 ± 1·27 S. E.	10·57 ± 0·90 S. E.
	Female	35	57·07 ± 0·25 S. E.	1·50 ± 0·18 S. E.	77·07 ± 1·49 S. E.	8·81 ± 1·05 S. E.

Food habits.—The Saurias and Santals are carrion eaters and have no objections to beef. The Mals, though having a common ancestral origin with the Saurias, have the strongest possible of prejudices against such foods. The *manjhi*

or headman in a Malpaharia village contemptuously observed to the author that the Saurias are barbarians as they partake of beef and that neither he (manjhi) nor any of his tribe would ever use any water for potable purposes from any *ghat* (dip in the hills) where even a cow has been found dead.

All these aboriginals indulge freely in strong drink. Though *haria* (an alcoholic beverage from rice, junera or other cereals) has been exempted from the operation of the excise laws and is prepared indigenously in the homes, yet it is said with a good deal of truth that vending of country liquor is the most lucrative business in the district. Apart from the Paharias who are noted for their thriftlessness the Santals also congregate in numbers near the village grog shop on *hatia* (market) days decked out in their fineries and accompanied by their friends for a carouse.

Rice and *makai* (maize) are the staple crop throughout the district and the other crop which supplements them is *junera* (great millet). particularly so in aboriginal homes. Both the Santals and the Paharias do not throw away the *marh* or conjee water after the cereals are cooked. Small amounts of *marua* (millet), *kurthi* (horse gram), *ghangra* (cow-pea), *kodo* and other millets are grown. Pulses are considered a luxury and not all can afford them. *Rahar* (red gram) and *kurthi* are the most popular of all pulses, and certain amount of *mung* (green gram) is also grown. (For botanical names *see* Table III.)

Any one of the above cereals boiled along with edible green leaves forms the chief and at times sole article of diet in Santal families. The Paharias store green leaves during autumn and winter seasons, dry some of them up (especially mustard leaves) and consume them gradually. *Saijan* or drumstick leaves (*Moringa oleifera*) are very popular. *Mahua* grows in abundance and when the flowers come out they are very soon shed by the tree, collected, dried in the sun almost like raisins, and used as staple food (cooked as any other grain food) late in summer and early in the rains, when the family store of other food grains get exhausted. Sometimes needy families cannot afford to wait for the *mahua* fruits to dry up but boil a part of their day's collection to a pasty consistency for immediate consumption. *Mahua* oil is also consumed in large quantities.

The use of milk and milk products as food is unknown among these aboriginal families. In the vicinity of big villages or towns, wherever some demand for milk exists, Santals do maintain milch cattle but they try to sell the milk to the last drop and whatever remains is made into butter or ghee and sold outside the village. The drinking of *mathha* (butter-milk) after fat had been extracted from the milk was never observed. But the aboriginal children (and at times the adults) have the healthy habit of munching green pods in a raw state as also various types of local fruits. The ' orange cup ' or the pink calyx of the marking nuts (*Semecarpus anacardium*) is considered to be a delicacy by the children.

The Paharia with his traditionally lazy habits displays a sort of abhorrence for cultivation. Consequently he has by experience discovered various edible roots and tubers which he digs out from the hills and makes flour out of them. Flour is also made out of the kernel of *kawach* or Cowage seeds (*Mucuna capitata*). The seeds are soaked in water, decorticated, the pulp boiled, thoroughly washed in water, dried and ground into flour.

Agriculture.—The principal crops in the district have already been mentioned. The respective raising seasons are shown in Table III. Besides these, the aboriginals (particularly the Santals) do maintain kitchen gardens yielding minor food crops or green vegetables. The agriculturist in the district suffers from a very great handicap due to scarcity of water.

TABLE III.

Principal crops used as food.

Hindustani name.	Botanical name.	Sowing season.	Harvesting season.
Chawal	*Oryza sativa*	June	November.
		July	December.
Makai ..	*Zea mays*	June	September.
Junera	*Sorghum vulgare*	June	November.
Kurthi	*Dolichos biflorus*	July	October.
Kodo	*Pasparum scrobiculatum*	August	December.
Marua	*Eleusine coracana*	June	September.
Mung	*Phaseolus radiatus*	June	September.
Ghangra	*Vigna catiang*	July	November.
Rahar ..	*Cajanus indicus*	July	December.
Sutari* ..	..	July	December.
Mahua	*Bassia latifolia*	.	From March.

* A smaller type of cow-pea, the botanical name not known.

Economic status.—A detailed economic inquiry was beyond the scope of the present investigation. Since the economic condition of any population group very largely influences their dietary, an attempt was made by a rough and ready method to assess the gross annual income of the families surveyed. The income is mainly derived from agriculture but in the great majority of cases it is supplemented by earnings from other occupations, such as stone-grinding, domestic labour, agricultural labour, building construction labour, etc. The dwellers on the hills (Paharias) as a rule collect dry faggots from the jungles and sell them in the neighbouring markets on the plains. The income accruing from agricultural produce

in each family has been estimated in accordance to the local market rates existing in 1938. The difficult part of the task was to calculate the earnings from daily wages as labour, because none of the people maintained any records whatsoever to show the number of days they were actually in employ during the year. After a good deal of local inquiry such income was calculated on the bases of employment for 300 days, 250 days, 200 days, 150 days and 100 days depending on the (*a*) amount of land in cultivation by the family (either as 'cultivating owners' or 'tenant cultivators'), (*b*) the number of persons in the wage earners' group and (*c*) demand for such labour in the neighbourhood. In converting the total income of each family into income per consumption unit the League of Nations' scale of family co-efficients (prescribed for calorie requirements) has been used in the absence of a better scale. The frequency distribution of the families according to their total annual income in rupees per consumption unit has been shown in the *Appendix*.

Families surveyed.

During the period October 1938 to January 1939 a dietary survey was carried out by the author in both the *damin* and non-*damin* tracts of the district. Altogether the food intake of 221 families consisting of 1,163 persons was investigated for periods of ten consecutive days, on the lines suggested by Aykroyd and Krishnan (1937). The Santal families sampled were residents of seven villages. In one particular village along with these Santal families a few Mahali and Dôm families were also included, as they were close neighbours. Both these tribes are semi-aboriginal in origin, the former earning their livelihood by weaving baskets from split bamboo and the latter by daily labour and occasionally as 'drummers' of the village music party on ceremonial occasions.

The Malpaharias were sampled from four villages and the Sauriapaharias from three villages situated within Damin-i-koh. All these Paharia villages are situated on the hill sides and tops. The Sauriapaharia families were surveyed last of all. The classification of families surveyed in the different groups is given in Table IV. In future for reference the group numbers only will be stated. For calculation of consumption units the indices prescribed by the League of Nations were used.

It appears from the last column of Table I that the total intake of calories is inadequate in all the groups, judged by any standard whatsoever. Of all the seven groups only IV and VII approach the barest minimum, whereas the families in group VI may be said to be living on actual starvation diets. It must be recorded that the survey was started at a time when the stores of grain foods in these families had become exhausted and the great majority were depending on wages earned in daily labour, more so in the case of the families in group I. The males were usually paid three annas every day and the females were paid either two or two and a half annas. The survey of group VII was carried out at a time when not only the cereals but also the legumes (such as *rahar*, *sutari*, *ghangra*) had been harvested; hence the higher calorie consumption. Except in the Mahali and the Dôm families the average number of consumption units is almost the same.

Table IV.

Classification of families surveyed.

Groups.	Name of villages.	Communities to which families belong.	Total number of families surveyed.	Total number of persons in families.	Average number of persons per family.	Number of consumption units.	Average number of consumption units per family.	Annual income per consumption unit (app.).	Average intake of calories.
								Rs. As.	
I	Bhaga ..	Santal	52	279	5·4	214·6	4·1	24 3	1,710
II	Mahulbana . Amui ..	..	36	198	5·5	151·3	4·2	25 7	1,954
III	Kushchira .. Amrapara ..	..	38	209	5·5	163·6	4·3	22 4	1,970
IV	Mahulbana ..	Mahali	7	27	3·9	21·5	3·1	36 1	2,275
V	do. ..	Dom	5	23	4·6	15·8	3·2	28 7	1.999
VI	Gomopahar .. Boropahar .. Ghoghridih .. Amarjwala ..	Malpaharia	39	214	5·5	159·5	4·1	15 5	1,368
VII	Jalokuri .. Chota Pakharia Sidhghat ..	Sauria-paharia	44	213	4·8	173·0	3·9	16 9	2,178
	Totals ..	..	221	1,163	..	..	..	..	..

Types of food consumed.

It has already been stated that these aboriginals have very little objection to the eating of meat. But from observation made during the diet surveys and visits to the various *hatias* (village markets) one can safely conclude that meat is a rare commodity and consumed only at very long intervals and during tribal hunts. A few Sauria families consumed beef. Table V gives the average intake of the various types of food in each of the groups. Fats and oils were rarely consumed, being costly articles of diet, and in no case was the intake of ghee recorded. The increased consumption of leafy vegetable in Santal families in comparison with that of the Paharias and Dôms, is quite evident. Regarding the cereals it may be noted that Santal and Malpaharia families consumed chiefly home-pounded parboiled rice and *makai* (maize). The Sauriapaharia families were found to depend mainly on great millet or *junera*. The intake of milk or milk products was negligible in all the groups. No sugar or jaggery was consumed by any of the families during the periods of investigation.

Table V.

**Average intake of the different types of food in ounces per day in the different groups.*

Group numbers.	Cereals.	Pulses.	Leafy vegetables.	Non-leafy vegetables.	Fats and oils.	Flesh foods.	Milk and milk products.	Fruits and nuts.
I	16·2	0·4	2·0	0·5	Neg.†	0·2	0·2	Neg.
II	20·1	0·6	2·0	0·5	0·1	0·1	0·1	Neg.
III	19·3	0·2	1·6	0·8	*Nil*	Neg.	*Nil*	*Nil*
IV	20·4	0·8	1·6	0·7	0·2	0·4	*Nil*	*Nil*
V	18·4	1·1	0·3	0·5	Neg.	Neg.	*Nil*	*Nil*
VI	13·1	0·5	0·9	0·3	*Nil*	*Nil*	*Nil*	*Nil*
VII	14·9	3·9	0·4	0·2	0·1	0·3	*Nil*	Neg.
Averages ..	17·5	1·1	1·3	0·5	*Nil*	0·1	*Nil*	*Nil*

* Detailed data may be had on reference from the author.
† Less than 0·1 oz.—Neg., i.e., negligible.

Proximate principles of food.

Protein.—The intake of protein was extremely inadequate except in the case of Sauriapaharias. The quota of animal protein was negligible. It is admitted that a dietary survey covering a period of 10 days during one particular season of the year, especially during a season when the flow of income is at a low ebb, has its defects, but certainly the people are not regular meat eaters. The Saurias mainly consumed beef. The majority of Santals in groups I, II and III were found to be supplementing their very modest meals with fish caught in neighbouring water-courses. The chickens from the poultry-yard rarely found their way into the stomachs of their owners.

TABLE VI.

** Average intake of proximate principles of food in grammes.*

Group numbers.	Protein (g.).	Percentage of animal protein.	Fat (g.).	Percentage of animal fat.	Carbohydrate (g.).	Calcium (mg.).	Phosphorus (mg.).	Vitamin A, I. U.	Vitamin B, I. U.	Vitamin C (mg.).	Percentage of calories from cereals.	Percentage of calories from pulses.	Percentage of calories from fats.
I	45·9	2·3	5·3	3·5	368	0·38	1·41	3,001	466	114	94·1	2·0	0·6
II	50·2	1·1	6·8	2·5	387	0·37	1·50	2,807	506	99	93·6	2·7	1·0
III	50·6	0·1	2·7	0·8	469	0·31	1·54	2,329	559	84	97·3	0·7	*Nil*
IV	55·7	3·2	7·6	4·5	473	0·34	1·55	2,594	618	73	91·7	2·6	2·3
V	52·1	0·2	4·0	0·2	441	0·23	1·54	520	570	18	92·9	5·7	0·5
VI	37·3	*Nil*	3·5	*Nil*	295	0·16	1·10	1,143	366	45	95·2	2·8	*Nil*
VII	76·0	1·7	13·9	3·0	422	0·47	1·70	983	274	24	80·8	15·8	0·9
AVERAGES	52·4	1·2	6·3	2·1	408	0·32	1·48	1,911	480	65	92·2	4·6	0·8

* Detailed data may be had on reference from the author.

Calcium.—The principal and almost sole source of this element in the dietary surveyed was the leafy vegetable. In the case of a few Sauria families *sutari* contributed more than 80 per cent of the total intake of this element. It has been found by the author (unpublished results) that *sutari* is a very rich source. The intake of calcium is much below the recognized minimum level.

Phosphorus.—If the standard laid down by Sherman (1937) be accepted (i.e., 1·32 g. per day) then except in group VII the intake of this element was adequate. The major portion of phosphorus consumed was, however, obtained from vegetable food.

Vitamins.—Edible green leaves are very popular with the Santals. Consequently vitamin-A intake was fairly high except in the Paharia and Dôm families. It may be said that Santals with slight effort are utilizing this gift of nature to its fullest advantage as compared with the other aboriginals. The adequacy or otherwise of vitamin C closely follows that of vitamin A in the table.

With home-pounded parboiled rice as staple article of diet there seems to be slight or no risk of vitamin-B_1 deficiency in the dietary. The use of milled cereal is unknown. Further, the water in which these cereal grains are boiled is not thrown away. The intake figure of this vitamin in group VII seems to be inadequate but it must be noted that the vitamin-B_1 content of *sutari* (legume) is not known and hence could not be included in the total figure.

In converting the raw foods used in the kitchens into the proximate principles of food the tables of food values in Health Bulletin (1938) and in the publication by the author (Mitra, 1938 ; Mitra *et al.*, 1940) on the subject were used. In some cases the food values have been worked out chemically in the author's laboratory (results unpublished).

Discussion on the improvement of the dietary.

Quantitative.—Making sufficient allowance for the fact that the dietary survey was conducted at the time of the year when the family stores of grains had been completely exhausted and that the aboriginals are of a comparatively light build, it is evident, from the data presented, that the food intake in all these seven groups of population was inadequate (hopelessly so in some cases) both in quantity and quality. The consumption of food quantitatively inadequate by any group of population for any length of time obviously implies extreme poverty. This financial stringency may be either due to inadequate income in the groups of the population under investigation or to an indebtedness which swallows the small income accruing from modest agricultural pursuits or/and wages from daily labour.

A detailed economic inquiry was not attempted. But the impression was gained that a large amount of indebtedness does exist. *No improvement in the dietary is possible unless preceded by betterment of economic status.*

The abolishment of the vice of drunkenness (leaving aside the domestic manufacture of *haria*) would be sure to improve the standard of dietary. With better facilities for agriculture, and suitable propaganda, the yield of crops would be likely to increase. It would be easier to tackle a Santal than a Paharia in matters involving manual labour. The solution of the problem of indebtedness deserves a careful

study. Lastly, it may not be out of place to suggest that steps should be taken to protect simple and illiterate folk habitually given to thriftlessness from the wiles of unscrupulous traders and money-lenders in monetary transactions.

Qualitative.—In the presence of gross qualitative deficiency in the dietary the considerations for qualitative improvement must be relegated to a secondary position.

From the trend of dietary habits among the Santals observed during the survey, it can be safely presumed that increase in income would improve the qualities of diet automatically in the group. This follows their liking for leafy vegetables, legumes and meat. The Paharias would need ceaseless persuasion and propaganda before they would agree to any modification in dietary habits.

Nutrition survey of children.

(*a*) *Anthropometric.*—During the diet survey operations 2,007 aboriginal children were examined as to their height and weight measurements, clinical assessment of the state of nutrition and incidence of deficiency conditions. For the purposes of the study the Malpaharia and Sauriapaharia children have been classified as one group. Sarkar (1935-36), who has made an intensive study on the racial affinity of both the groups, is of opinion that they have sprung from common ancestors. As stated earlier the Paharia villages contain very small numbers of people

Table VII.

Average height in inches of aboriginal children in different age groups.

Age in years.	Santals.				Paharias.			
	Boys.		Girls.		Boys.		Girls.	
	Number examined.	Height.	Number examined.	Height.	Number examined.	Height.	Number examined.	Height.
3	42	31·5	28	30·5	10	30·9	12	28·2
4	54	34·7	48	33·5	20	34·5	17	33·4
5	64	37·5	67	36·2	21	37·0	17	36·8
6	76	39·8	60	39·3	35	40·4	30	39·3
7	92	42·4	73	42·0	36	43·5	34	42·4
8	99	46·0	81	45·1	40	45·5	37	45·3
9	98	47·4	69	46·9	25	47·6	19	47·2
10	99	49·9	40	49·2	45	49·0	21	48·8
11	97	52·0	58	51·5	41	51·8	16	50·1
12	85	54·6	36	52·7	17	54·0	11	52·0
13	46	56·1	21	54·4	23	55·5	6	53·8
14	30	59·0	*Nil*	..	11	56·4	*Nil*	..
Totals ..	882	..	581	..	324	..	220	..

(consequently children) and are situated on hill tops and sides at a considerable distance from one another. This accounts for the smallness of the number of Paharia children examined. The heights and weight for different groups are shown in Tables VII and VIII. The tables taken at their face value give the impression that in the same age group Santal children are usually heavier and taller than the Paharia children.

TABLE VIII.

Average weight of aboriginal children in pounds in different age groups.

Age in years.	Santals.		Paharias.	
	Boys.	Girls.	Boys.	Girls.
3	22·7	21·1	22·1	20·6
4	25·6	25·3	25·3	24·9
5	29·2	27·0	27·8	26·2
6	33·9	30·1	30·8	29·8
7	36·9	34·7	34·5	34·1
8	40·9	39·8	40·6	39·3
9	44·9	43·5	43·5	43·3
10	50·3	48·6	49·3	46·1
11	56·7	53·7	55·2	53·1
12	61·5	60·1	61·0	57·7
13	66·9	64·3	64·5	63·9
14	76·0	..	74·5	..

(*b*) *State of nutrition.*—The children were examined clinically on the lines described by the author (Mitra, 1940) and rated as 'good', 'fair' or 'poor'. The percentage incidence with poor nutrition was found to be higher among the Paharia

children than among the Santal children. Table IX gives the results of clinical rating by naked-eye examination. In spite of the drawbacks this method has its uses and is still being used by the School Medical Officers in Great Britain.

TABLE IX.

Incidence of the state of nutrition amongst aboriginal children.

Rating.		Santals. Boys.	Santals. Girls.	Santals. Total.	Paharias. Boys.	Paharias. Girls.	Paharias. Total.
Good	Actual ..	124	73	197	13	7	20
	Percentage ..	14·0	12·5	..	1·0	3·2	..
Fair	Actual ..	611	410	1,021	226	158	384
	Percentage ..	69·3	70·6	..	69·8	71·7	..
Poor	Actual ..	147	98	245	85	55	140
	Percentage ..	16·7	16·9	..	26·2	25·1	..

(c) *Deficiency conditions.*—Aykroyd and Rajagopal (1936) emphasized the importance of the incidence, the signs of deficiency diseases, particularly xerophthalmia, phrynoderma and angular stomatitis and their correlation with the state of nutrition. Table X gives the findings during the present survey of the conditions

TABLE X.

Percentage incidence of the physical signs supposed to be associated with malnutrition amongst aboriginal children.

Race.	Sex.	Total number examined.	(a) Xerophthalmia.	(b) Phrynoderma.	(c) Angular stomatitis.	(d) Malocclusion.	(e) From (a), (b) or (c).
			Percentage found to be suffering from :—				
Santal ..	Boys ..	882	3·1	6·8	1·5	29·1	11·8
	Girls ..	581	2·8	5·9	1·0	28·6	9·3
Paharia ..	Boys ..	324	2·8	28·7	*Nil*	19·4	29·9
	Girls ..	220	2·3	24·6	*Nil*	23·2	26·8

enumerated in columns (*a*) to (*e*). No suggestion supported by scientific findings have yet been made to show that malocclusion of teeth is caused by malnutrition. But such figures have recently been included by nutrition workers (Wilson and Mitra, 1938 ; Mitra, 1939 ; Shourie, 1939 ; Singh, 1939) in their reports to find out whether this condition has any association whatsoever with malnutrition.

Phrynoderma supposed to be associated with inadequate intake of vitamin A was more common among Paharia children. It has been shown that Santals consume more of vitamin A than the Paharias (*see* Table VII).

On the whole it may be stated without reservation that during this investigation Santal children were found less malnourished than Paharia children.

SUMMARY.

1. The diet of 221 aboriginal families in the Santal Parganas (district of Bihar) has been investigated for a period of 10 days during the cold weather season.

2. Mean intake of calories in the various groups varied from 1,400 to 2,200. The diet of the majority was grossly deficient in quantity even when allowance is made for the small stature of the population concerned. The diet also showed serious qualitative defects since over 90 per cent of total calories was obtained from cereals.

3. One group (Santals) maintained vegetable gardens and consumed leafy vegetables in fair quantities. Consequently their intake of vitamins A and C was higher than that of other groups (Paharias) not producing vegetables in the same amounts. The Paharias inhabit the hill tops and sides, whereas the Santals live in the more fertile valleys.

4. Two thousand and seven children were examined. The Paharia children were smaller and lighter than the Santal children and showed a much higher incidence of deficiency diseases.

5. The gross poverty of these communities makes any improvement in diet difficult.

ACKNOWLEDGMENTS.

To Mr. F. Rowat, of the Mission at Bhaga, the author is indebted for the help and facilities of work at Bhaga. The author is also obliged to Mr. S. S. Prasad, Divisional Forest Officer, for help and assistance rendered to the field workers by forester Babu Srikrishna Sahai. He is also obliged to his assistant Dr. N. P. Varma, for all assistance in the examination of children. To his chief Lieut.-Colonel S. L. Mitra, I.M.S., the author is grateful for his advice and encouragement, and to Dr. W. R. Aykroyd, Director, Nutrition Research, Coonoor, for his valuable suggestions in revising the manuscript.

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APPENDIX.

Frequency distribution of the families according to their total annual income in rupees per consumption unit.

Annual income.	Group I.		Group II.		Group III.		Group IV.		Group V.		Group VI.		Group VII.	
	Frequency.	Percentage.	Frequency.	Percentage.	Frequency.	Percentage.	Frequency.	Percentage.	Frequency.	Percentage.	Frequency.	Percentage.	Frequency.	Percentage.
Rs. 5	0	..	0	..	0	..	0	..	0	..	8	20·5	3	6·8
Rs. 10	6	11·5	0	..	4	10·5	0	..	1	20·0	8	20·5	17	38·6
Rs. 20	19	36·5	12	33·3	18	47·4	0	..	1	20·0	13	33·3	12	27·3
Rs. 30	13	25·0	17	47·2	10	26·3	1	14·3	2	40·0	7	17·9	5	11·4
Rs. 40	8	15·4	4	11·1	2	5·3	3	42·9	0	..	2	5·1	4	9·1
Rs. 50	4	7·7	1	2·8	2	5·3	3	42·9	0	..	0	..	2	4·5
Rs. 75	2	3·8	2	5·6	1	2·6	0	..	1	20·0	1	2·6	1	2·3
Rs. 100	0	..	0	..	0	..	0	..	0	..	0	..	0	..
Over Rs. 100	0	..	0	..	1	2·6	0	..	0	..	0	..	0	..
Totals ..	52	99·9	36	100·0	38	100·0	7	100·0	5	100·0	39	99·9	44	100·0

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THE OPTIMUM REQUIREMENTS OF VITAMIN C OF PERSONS LIVING ON A BENGALI DIET.

BY

N. M. BASU,

AND

G. K. RAY.

(An Inquiry under the Indian Research Fund Association.)

(From the Physiological Laboratory, Presidency College, Calcutta.)

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THE daily requirements of vitamin C have been studied in Europe and America. According to Hawk (1938) infants require 5 mg., adults 15 mg., and pregnant and lactating women 25 mg. daily. Schültzer (1937) considered 40 mg. of vitamin C to be the daily requirements, as he found that the daily intravenous injection of this amount proved to be a curative dose for a patient with moderate scurvy. As the dietetic habits of Bengalis differ widely from those of Europeans and Americans, it is possible that their requirements of this vitamin would be different from those of others. The present enquiry was, therefore, undertaken with a view to determine, if possible, the optimum requirements of this vitamin by Bengalis.

PROCEDURE.

Various methods have been employed for determining the daily human requirements of vitamin C and they have yielded different results. Thus, by using the capillary resistance test, Gothlin (1931) concluded that 40 c.c. to 60 c.c. lemon juice (i.e., 20 mg. to 30 mg. vitamin C) are required to protect a person from scurvy. Van Eekelen (1936) determined the daily human requirement by giving large doses of ascorbic acid, 250 mg. to 500 mg. daily, until saturation was attained. The total amount of ascorbic acid necessary for reaching the state of saturation, divided by the number of days required, was taken as the daily requirement. Other workers like van Wersch (1936, 1936*a*) and Heinemann (1936) determined the daily requirements on the same principle. Schültzer (*loc. cit.*) showed that this method

of calculation is untenable as the total quantity of ascorbic acid necessary to reach saturation depends upon the size of the daily dose given. Schültzer (*loc. cit.*) has shown by the intravenous injection of ascorbic acid, as mentioned before, that 40 mg. of vitamin C or perhaps a slightly smaller dose, is the daily human requirement.

The writers approached this question from a different aspect and tried to obtain the daily optimum requirements of this vitamin in the following way :—

Persons were brought to the saturation state by repeated daily doses of the vitamin till the excretion of the excess intake became fairly constant, the normal intake of the vitamin through the diet being kept, as far as possible, low and constant during the period of the experiment. The excess ingestion was then reduced until that stage was reached at which the excess excretion remained constant for several consecutive days at nearly 30 per cent of the excess intake, indicating the lower limit of saturation of the body (Youmann, 1937). The amount of excess intake of the vitamin at this stage plus the small amount of the same, present in his diet, has been taken to be the optimum intake required daily by the person for keeping his body at a lower state of saturation. The reasons for this suggested procedure are as follows :—

The optimum requirement of the body of any dietary essential would necessarily be such an amount as would not only meet the normal requirements of the body but be quite adequate for satisfying the extra demands of the body under emergency conditions. Although the extra demands of the body for this vitamin under various conditions cannot be ascertained, yet it is obvious that the intake of that amount of the vitamin which would keep the body at a uniform (though partial) state of saturation, would leave behind sufficient store of the substance in the body to be drawn upon or utilized during emergency. To make the body uniformly but partially saturated at the shortest possible time, it was found necessary to saturate it quickly and fully and then reduce the saturation to the desired level.

Experimental.

The method of estimation with the indophenol dye was adopted. Redoxon Roche tablets (i.e., standardized vitamin C tablets of Hoffmann la Roche) were used in these experiments as supplement.

The values of vitamin C in the cooked diets of the subjects were calculated from the data obtained in the Laboratory in the course of estimation of losses of vitamin C in various vegetables during cooking. The average composition of the diet of these subjects was as follows : Rice 280 g., whole wheat 70 g., vegetables (mainly potatoes) 200 g., pulses 50 g., milk—variable (225 g. to 900 g)., fish 50 g., some spices and sweets. The total carbohydrate content of this diet is over 300 g.

The subjects kept their diets, as far as practicable, constant during the period of the experiments, the vitamin C content being low.

These experiments were performed on six subjects each of whom with one exception was brought to the state of saturation by a large daily intake of vitamin C tablets, as mentioned above, before their ingestion was reduced.

Results.

The results of these experiments are given in the Chart which gives a graphical representation of the percentage of excess excretion of vitamin C of the experimented persons after the intake of 50 mg. and also of 25 mg. in such cases only in which the excess excretion after the intake of 50 mg. was much above 30 per cent.

Graph 1 relates to subject K. P. P. whose average excess excretion of vitamin C for 5 days just previous to this experiment on optimum requirements was only 45 per cent of the intake, in spite of his getting daily a supplement of 500 mg. of ascorbic acid for 8 consecutive days. Before the present experiment was commenced he was thus given 4,000 mg. of ascorbic acid, yet his rate of excretion did not go above 45 per cent on the average. It appears from this that the rate of destruction or utilization of ascorbic acid or both in his body was very high. The subject was taking a large amount of vegetables. When his intake of vitamin C was suddenly reduced from 500 mg. to 50 mg., there was a considerable rise in the percentage of excretion on the following 2 days accompanied by a very abrupt fall (viz., from 44 per cent before the reduction of the intake to 66 per cent after the reduction and thence to 13 per cent). The significance of this fluctuation will be discussed in connection with Graph 4 which is analogous to this graph in various respects. It would appear from the graph that at an excess intake of 25 mg., the excess excretion of the subject became constant at 34 per cent which is a little above the lower limit of saturation.

Graph 2 shows very clearly the optimum requirement of the vitamin of subject A. K. D. The abrupt rise on the 4th day was due to the intake of extra vitamin in the form of fruits by the subject on the previous day. The graph shows that with the intake of 50 mg. there was a higher rate of excretion (appreciably above 30 per cent) and when the intake was reduced to 25 mg., the rate of excess excretion went down to a constant level of 31 per cent, which is the lower limit of saturation of the body.

Graph 3 relates to subject A. R. whose average rate of urinary excretion of free ascorbic acid, before this experiment was started, was only 2·5 mg. per day. After the administration of 2,000 mg. of ascorbic acid, his rate of urinary excretion rose to only 34 per cent of the excess intake. To find out what amount of ascorbic acid he would require at this state of saturation of his body for his daily optimum requirements, he was not given further doses of ascorbic acid for full saturation of his body. The graph shows that he required in addition to the amount of vitamin C present in his diet a daily dose of 50 mg. which is double the amount required by former subjects.

Graph 4 is interesting in various ways. This subject B. D. had an average daily urinary excretion of free ascorbic acid of 1·5 mg. only before this experiment. A large dose of ascorbic acid was daily administered to him till his urinary excretion of excess intake became very high and constant, viz., 81 per cent. At this stage his intake was reduced to 50 mg. There were considerable fluctuations in the rate of excretion of the vitamin, as in the case of K. P. P. in Graph 1. On the 1st day

there was considerable rise, then a marked fall and again a rise in excretion, although the intake was the same. This was also the case to some extent with K. P. P. Both these subjects were given daily a large dose of the vitamin for a longer period than others before their intake was suddenly reduced to 50 mg. In both cases, it is found, as pointed out before, that after the intake was abruptly reduced there was a marked fall in excretion either on the next day or the day after and then an appreciable rise replaced by an appreciable rise on the following day.

Thus, the fluctuations in the rate of excretion of the vitamin after a sudden reduction in intake are as follows :—

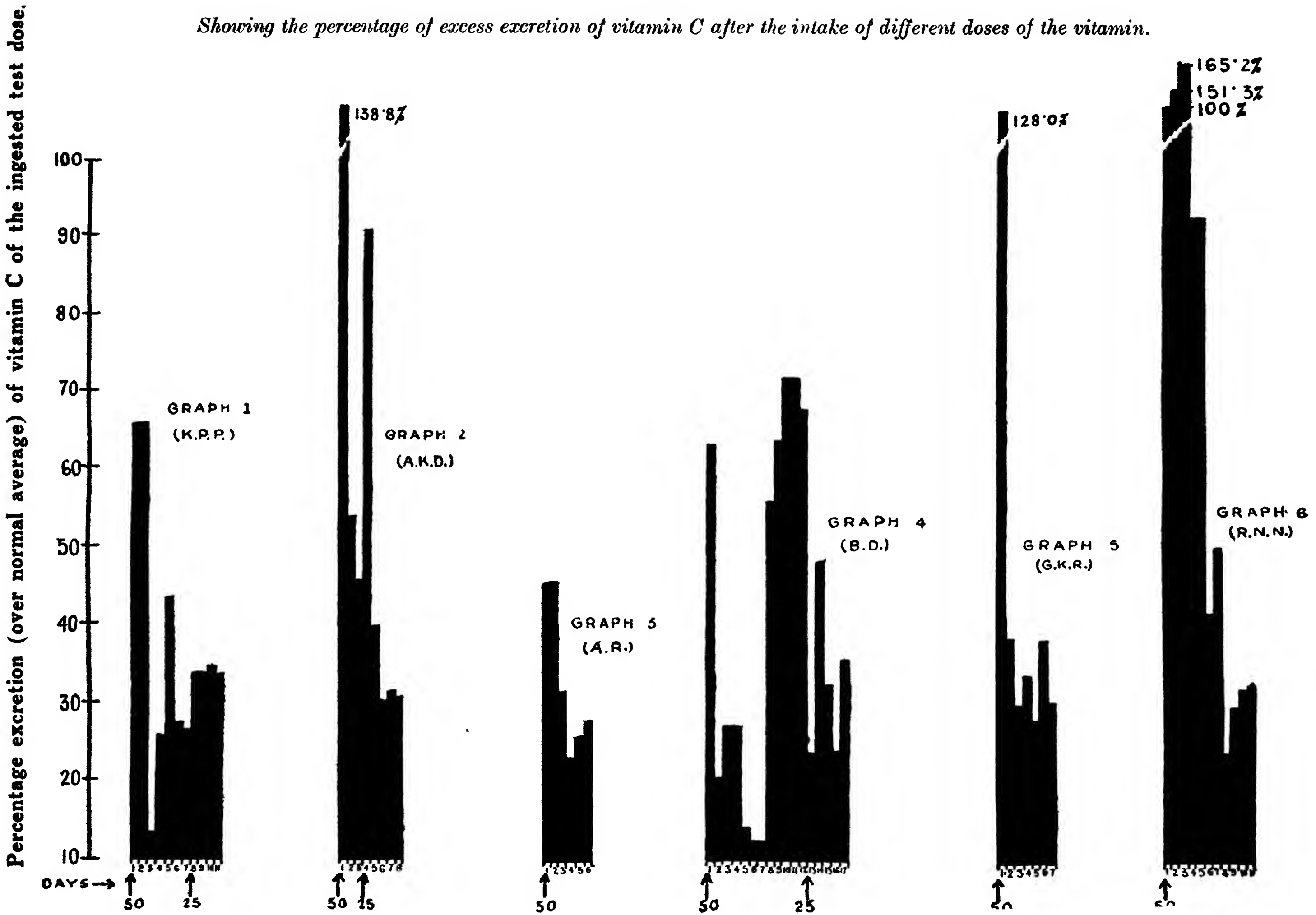
First, there is a sudden and appreciable rise in the percentage output of the excess intake. This is noticed in all cases. Following the rise there may be either a very great reduction in output for a day or two and then a rise to a more or less constant level of excretion, or an appreciable but proportional reduction in output which after slight fluctuations attains a more or less steady state (*cf.* Graph 4). Of these two subsequent phases the former is noticed in cases of such persons as ingested large doses of the vitamin for a pretty long time, as already pointed out. The first rise in the percentage output is, as has been discussed below, due to delayed excretion of the vitamin. The subsequent fall in percentage excretion is expected as the intake is reduced. The very great and disproportional reduction in output in certain cases is probably brought about in the following way : If a person be made to ingest large doses of ascorbic acid for a pretty long time and if the ingestion of such large doses be continued even after his body is saturated. the excess ingestion (i.e., the amount in excess of the daily requirements of the body) is probably disposed of quickly by the body either by irreversible oxidation or in some other way, while a large portion is being continuously eliminated through urine and sweat. This is apparent from the fact that, the percentage of elimination even during complete saturation of the body seldom exceeds 80. If the intake be abruptly reduced at this stage. but not below the normal requirements, the habit of increased disposal of the vitamin incurred during the condition of its supply in enormous amount, is not immediately given up but persists for a while till it is modified in accordance with the new condition of the reduced supply. The persistence of this habit is probably the cause of the very great and disproportionate reduction in excretion of the vitamin, particularly when the ingestion of large doses of the vitamin continues for a sufficiently long time to allow this habit to be established.

Similar phenomena are also noticed in connection with a sudden reduction in protein intake. Thus, if the protein ration of an animal be suddenly reduced from a high to a low dose, the disposal of protein by the body is not altered immediately, insomuch that there is loss of protein from the body at the 1st stage and then gradually the new N-equilibrium is attained.

Subject B. D. required like (1) and (2) an extra dose of 25 mg. only of vitamin C for his daily optimum requirement. His body, unlike the former subject, was allowed to be saturated before experiments on optimum daily requirements were performed.

Graph 5 relates to G. K. R. who ingested previously 1,670 mg. of crystalline ascorbic acid and 330 mg. of vitamin C from pine-apple juice in 4 days before the

Showing the percentage of excess excretion of vitamin C after the intake of different doses of the vitamin.



present experiment was started. The percentage excretion of the excess intake on the 4th day was 106·8. This shows clearly that a part of this day's excretion of vitamin C was derived from the previously ingested vitamin; in other words, there was delayed excretion of the vitamin ingested on the previous day. On the 5th day his intake was suddenly reduced to 50 mg. and the percentage excretion was 128, indicating again delayed excretion of the vitamin ingested on the previous day. The excretion then fell down without showing much fluctuation as in Graphs 1 and 4. The ingestion of large doses of the vitamin was not continued for a long period as in cases 1 and 4, mentioned before, and accordingly fluctuations were not expected and were not also obtained. He required 50 mg. of vitamin C in addition to what was present in his daily diet for maintaining his body at the lower state of saturation. Up till now it was noticed that when the body was fully saturated, an extra dose of 25 mg. of ascorbic acid sufficed for his optimum requirements. Accordingly G. K. R.'s case appears to be an exception. But when the amount of vitamin C present in his diet is compared with that of others, it will be observed that it is definitely less than theirs excepting A. R. and R. N. N. (Graphs 3 and 6) who were also getting a small supply of vitamin C from his daily diet (*vide* Table I).

Graph 6 gives the course of excretion of R. N. N. He was given a high dose of ascorbic acid (250 mg. daily) for 7 days (first 3 days—orange juice, sufficient amount to contain daily 250 mg. vitamin C, and the last 4 days standardized tablets containing the same amount of vitamin C each day), at the end of which the percentage of excess excretion of the vitamin was 70. While his body was thus saturated, his intake was cut down to 50 mg. On the 1st day of his reduced intake the percentage of excess excretion was very high as was noticed in all these cases, being probably due, as suggested before, to the delayed excretion of the large dose of the vitamin ingested on the previous day. The excretion for the next 3 days was also very high. This was due to his secret intake of fresh fruit juice for 2 days. The excretion then fell down with slight fluctuations to a more or less constant level of nearly 32 per cent. Thus this subject, in spite of the previous saturation of the body, required also 50 mg. of ascorbic acid in addition to what was present in his diet for maintaining his body at the lower state of saturation. The amount of vitamin C present in his diet was only 18·1 mg., i.e., as low as that of other subjects who also required the same amount of excess intake.

Daily requirements of vitamin C in mg. as calculated from the results obtained.

Table I gives the various figures from which the actual daily requirement of vitamin C in mg. for maintaining the body of each of the experimented persons at the lower state of saturation, has been calculated. It is seen that the average actual daily requirement is 44·2 mg. and the average daily intake to meet this requirement is found to be 66·2 mg., i.e., roughly about 70 mg.

Table II gives the actual requirement of vitamin C per lb. weight of the body and the actual intake per lb. weight to meet this requirement. The average requirement per lb. weight is 0·36 mg. and the average intake necessary to meet this requirement per lb. weight is 0·552 mg.

Discussion.

The figures for the daily requirements of vitamin C, as arrived at by the various methods suggested by different workers in Europe and America where the diet of people is predominantly protein-rich, are not uniform but varying from 20 mg. to 60 mg. Thus, according to Gothlin (*loc. cit.*) they are 20 mg. to 30 mg. Hamel (1937) suggests 40 mg. and Schültzer (*loc. cit.*) also came to the same conclusion. Van Eekelen (*loc. cit.*) thinks it to be 60 g. for a man weighing 70 kg. and Heinemann (*loc. cit.*) obtained a similar result, viz., 60 mg., while Wachholder (1938) thinks that the daily intake shall not fall below 50 mg. if the body deficit be not made to exceed 500 mg. to 600 mg. Kellie and Zilva (1939) consider that 15 mg. would be sufficient not only to prevent scurvy but to maintain an individual in good health.

The present investigation showed that the actual average requirement is 44 mg. per day. As the average daily loss through urine of the ingested vitamin is 22 mg. the daily intake should amount to 44 plus 22, i.e., 66 mg., or roughly 70 mg. It is difficult to say what proportion of the vitamin calculated to be actually required by the body is utilized therein and what proportion is wasted away. That a portion of this 44 mg. is lost through sweat, the amount thus lost increasing during copious perspiration, is beyond doubt (Wright, 1936; Bernstein, 1937). It is permissible to assume that a further portion of this vitamin may be lost in the bladder where the urine remains stored for some time before it is voided, particularly when the urine secreted is alkaline or neutral or faintly acid in reaction. As the persons who were examined in the course of this experiment were taking a considerable amount of leafy vegetables which leave behind an alkaline ash, it is justifiable to assume that the urine secreted by them and stored in the bladder was at least not strongly acid. Accordingly, a portion of the vitamin is liable to be destroyed, and the destruction is greater in amount the longer the urine remains stored in the bladder.

In view of these considerations it may be concluded that the amount actually required for utilization in the body is appreciably less than 44 mg. which is the calculated actual requirement of the vitamin. Thus, the real daily requirement of the vitamin in this country may not differ much from that obtained by workers of other places, or, in other words, the different dietetic habits of the people of this country and its different climate may not influence the actual utilization of the vitamin in the body, although, there are reasons to believe, they may cause an appreciable loss of the ingested vitamin.

After examining columns 9 and 10 in Table I it is observed that the average utilization of the vitamin in the body varies inversely according to its state of saturation. Thus, subject 4 who required the maximum amount of vitamin C to be saturated, was normally utilizing the maximum percentage of the intake of the vitamin. This also holds good in the case of subjects 2, 3, 5 and 6, but subject 1 appears to be an exception. This subject, as noticed before, was peculiar, for his excretion did not change for 5 days previous to these experiments, in spite of a daily intake of large doses of the vitamin, and there are, therefore, reasons to believe that the rate of destruction of the vitamin in his body

is greater than normal. Accordingly, the percentage of utilization of this vitamin is less than what is expected from the state of saturation of his body.

Further consideration of Table I shows that the actual daily requirements of vitamin C of subjects 3 and 4 are very nearly the same, viz., 50 mg. and 51·3 mg., respectively, although the former subject, i.e., subject 3, was in a lower state of saturation at the beginning of the present series of experiments, his excess excretion being only 34 per cent, when his ingestion was reduced to 50 mg. per day and the latter subject, i.e., subject 4, was in a completely saturated condition when the experiment was commenced (*vide supra*). It thus appears obvious that the actual daily requirement of vitamin C is independent of the state of saturation of the body, although the intake of the vitamin per day to meet this requirement may vary.

It has been mentioned before and has also been well brought out in the graphs, that the percentage excretion of the vitamin on the day following the suddenly reduced intake was very high and it was suggested that this was due to delayed excretion of a part of the vitamin ingested on the previous day. It must not be assumed therefrom that such delayed excretion takes place only, if the vitamin intake be suddenly reduced. It is quite likely that there is generally a delayed excretion every day to some extent, but while this delayed excretion of a few mg. does not raise the percentage excretion appreciably when the intake is large, it affects the latter (i.e., the percentage excretion) very significantly when the intake is suddenly reduced to a small amount. This is the reason why the percentage excretion on the day following the suddenly reduced intake of vitamin C mounted up in each case to a very high figure.

Kellie and Zilva (*loc. cit.*) think that a dose of 15 mg. would be sufficient to maintain an individual in good health, although they advised that for ensuring a margin of safety, 30 mg. to 40 mg. per day should be the requisite amount of vitamin C intake for healthy adults. It has been shown recently by various workers that the vitamin C content of plasma and the complement value thereof run parallel. If, therefore, the average complement value of plasma or serum of normal healthy persons be found out, the amount of vitamin C intake which would be required to maintain this average complement value of the serum, may be taken as the normal human requirement. Experiments are in progress in the Laboratory to determine the normal requirement of the vitamin in this way.

Summary.

1. Six persons were examined for their optimum requirements of vitamin C. Excepting one, all were given large doses of vitamin C till they were saturated according to the urinary test. The dose was then reduced to 50 mg. per day and their excess excretion was noted. If it was above 30 per cent, the dose was further reduced to 25 mg. which was continued for several days. If the average excretion was now found to be near about 30 per cent, then this dose plus the amount of vitamin C present in his diet, was taken to be his optimum requirement. The reason for this conclusion has been discussed in the text.

2. The actual daily requirement was found to be on the average 44·2 mg. and the actual daily intake of this vitamin to meet this requirement was calculated to be 66·2 mg.

3. If a person is given large doses of the vitamin for a long period, his body probably develops the power of destruction of the vitamin to a greater extent than under conditions of normal intake. This power generally disappears within 48 hours after the intake is considerably reduced.

4. The greater need of the vitamin in a moist and warm place is probably due to its loss from the body through copious sweat. An excess of carbohydrate in the diet does not apparently influence the utilization of vitamin C in the body.

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TABLE I.

Optimal intake of vitamin C per day.

Number.	Subject.	Average normal daily excretion of vitamin C (in mg.) in urine.	Average daily amount of vitamin C (in mg.) present in the cooked diets of the subjects.	Average daily utilization of vitamin C (in mg.) in the body of the subjects when on their usual diet (i.e., the amount of vitamin C taken daily with the diet minus its average normal daily excretion).	Average daily utilization of the added amount of vitamin C (in mg.) for maintaining the body at the lower state of saturation. (Excess amount of vitamin C ingested minus the average excess excretion.)	The total daily requirement of vitamin C (in mg.) for maintaining the body at the lower state of saturation.	Total vitamin C intake (in mg.) per day to meet the daily requirement (i.e., vitamin C present in the diet plus the amount supplemented).	Percentage of average utilization of vitamin C present in their normal diet.	Amount of vitamin C (in mg.) required for reaching the state of saturation.	Percentage of excess excretion when the state of saturation was reached.
	1	2	3	4	5	6	7	8	9	10
1	K. P. P.	19·0	39·1	20·1	25— 8·5. i.e., 16·5	36·6	(39·1+25), i.e., 64·1	51	1,599	56·2
2	A. K. D.	11·0	36·0	25·0	25— 7·8, i.e., 17·2	42·2	(36 +25), i.e., 61·0	69	1,536	73·4
3	A. R.*	4·0	18·0	14·0	50—13·5, i.e., 36·5	50·5	(18 +50), i.e., 68·0	78	1,664	34·0
4	B. D.	2·4	36·9	34·5	25— 8·2, i.e., 16·08	51·3	(36·9+25), i.e., 61·9	95	2,410	81·0
5	G. K. R.	11·6	24·3	12·7	50—16·5, i.e., 33·5	46·2	(24·3+50), i.e., 74·3	50	616	106·8
6	R. N. N.	12·1	18·1	6·0	50—17·6, i.e., 32·4	38·4	(18·1+50), i.e., 68·1	33	248	59·2

* Indicates Mohammedan student.

N.B.—The average daily requirement of vitamin C for maintaining the body at the lower state of saturation is $\frac{36{\cdot}6+42{\cdot}2+50{\cdot}5+51{\cdot}3+46{\cdot}2+38{\cdot}4}{6}$, i.e., 44·2 mg.

The average daily intake of vitamin C for meeting the daily requirement of our body for maintaining it at the lower state of saturation is $\frac{64{\cdot}1+61+68+61{\cdot}9+74{\cdot}3+68{\cdot}1}{6}$, .e., 66·2 mg.

The large variation in the amount of vitamin C present in the cooked diets of the subjects, as given in column 3, is due to a considerable difference in the nature of foods taken by these subjects. Each subject, except A. K. D., kept his own diet constant during the course of the experiment.

TABLE II.

Optimal intake of vitamin C per day per lb. weight of the body.

Number.	Name.	Weight in lb.	Mg. of vitamin C to be taken daily to meet the daily requirement of the body.	Mg. of vitamin C to be taken daily to meet the daily requirement per lb. weight of the body.	Mg. of vitamin C actually required per day per lb. weight of the body.
	1	2	3	4	5
1	K. P. P.	127·5	64·1	0·50	0·28
2	A. K. D.	120·25	61·0	0·50	0·35
4	B. D.	98·0	61·9	0·63	0·51
5	G. K. R.	140·0	74·3	0·53	0·33
6	R. N. N.	113·0	68·1	0·60	0·33

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STUDIES ON PEPTIC ULCER IN SOUTH INDIA.

Part I.

INTRODUCTION AND CLINICAL STUDY OF 258 CASES.

BY

MAJOR J. R. DOGRA, M.D., I.M.S.,

(Work done under the Indian Research Fund Association.)

(From the King Institute, Guindy, Madras.)

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I. INTRODUCTION.

IN spite of the progress in many branches of medical science in recent years, and the high incidence of gastric and duodenal ulcers throughout the world, very little new light has been thrown on their ætiology.

Dyspepsia has for long been known to be a common disease in South India. It had been considered primarily a medical condition and patients travelled from clinic to clinic swallowing quantities of alkalis without obtaining permanent relief. This state of affairs still exists in parts where either the facilities for investigation of cases of dyspepsia are not available or where the medical profession has not yet realized that dyspeptic symptoms are often the result of a lesion in the stomach or duodenum which required careful diagnosis and adequate medical or surgical treatment and not indiscriminate half measures. Writing on the infrequency and rarity of gastric and duodenal ulcers in India, Rogers and Megaw (1935) stated, ' This bears out the writer's experience in India and it may be related to the largely vegetarian diet of Indians in whom appendicitis is also considerably less frequently seen than among Europeans '.

Records show that Niblock (1905) performed the first gastro-enterostomy for duodenal ulcer in South India at the Government General Hospital, Madras. In 1912 Pugh (1913) at the London Mission Hospital, Neyyoor (South Travancore), performed gastro-jejunostomy for duodenal ulcer much against the then current medical opinion regarding this surgical procedure for ' dyspepsia '.

It was not, however, until 1922, when Somervell in Travancore and Bradfield in Madras recognized the great prevalence of 'dyspepsia' in South India and associated this symptom complex with pathological lesions in the form of ulcers in the stomach and duodenum, that large number of 'dyspeptics' were operated upon with apparently successful results. As a result, within a few years, the number of gastro-enterostomies performed at the Government General Hospital, Madras, and the London Mission Hospital, Neyyoor, increased considerably. It may be recollected that about this time Moynihan (1923) described the classical symptomatology of this disease before the Hunterian Society of London and drew the attention of the medical profession of the world to his experiences of ten years.

At the 7th Congress of the Far Eastern Association of Tropical Medicine held at Calcutta the surgeons in India presented their observations on a large series of operated cases with a view to elucidating the probable ætiological factor or factors responsible for the prevalence of peptic ulcers in South India. Their opinions differed greatly. Bradfield (1927) pointed out that 82 per cent of the cases had pyorrhœa alveolaris and that all the patients were grossly underweight. He drew attention to the diet of these cases pointing out its deficiency in vitamins and it was argued that 'this deficiency is shown by a degenerative hyperæmia and other changes in the appendix, while focal infection and infected teeth are the final agents which produce ulceration in the stomach or duodenum of lowered vitality'. With regard to constipation he stated: 'A theory of intestinal stasis will not explain the universality of the disease'. It may here be stated that in America, Rosenau (1916), working on non-hæmolytic *streptococci*, had developed a theory of the property of elective localization of these organisms and had produced experimental evidence to support the theory of 'focal sepsis' and its rôle in the ætiology of varied conditions including gastro-duodenal ulcers.

On clinical evidence at his disposal Bradfield (1927) stated, 'Our patients came from all races and castes. It is remarkable how closely the figures of the different population groups in census reports correspond with the hospital admission rates and the distribution of gastric ulcers. The disease is not confined to any one district in the presidency and the beggar, the coolie, the rich man, the scholar and the agriculturist are all numbered amongst its victims'.

Somervell (1927) on the other hand stated, 'Our patients do not correspond at all with the distribution of castes in the census, nor in our general hospital admissions'. Comparing the total number of duodenal ulcer cases with the percentage of total hospital admission he showed that duodenal ulcer was far more rare in Brahmins and far more common in Hindu out-castes. He showed the regional distribution of the disease and associated it with the consumption of tapioca as the staple food. He absolved betel chewing, hot spices and curries and bad teeth from playing any part in the ætiology of the disease and advanced the following theory: '(*i*) tapioca diet leads to (*ii*) chronic constipation which leads to (*iii*) appendicitis of a mild order tending to become chronic rather than acute and involving the lymphatic system draining the appendix region, by extension of which (*iv*) a duodenitis is produced, which may give rise to pyloric spasm and cause a few people

to seek relief from this, but more often leads to (*v*) a definite pyloric or duodenal ulceration, causing pain and spasm of the pylorus in the initial stages and stricture later. In some cases, no doubt, the appendicitis stage is missed out and the carbohydrate diet leads directly to duodenal ulceration, possibly preceded by a local duodenitis '. He explained further that ' tapioca diet leads so commonly to the series of events quoted, because it is deficient in vitamin B '.

About this time Hingston (1927), in Calcutta, analysed the hospital records of various provinces, including those of Madras, and, although statistics showed marked differences in the incidence of the disease, he explained that this marked discrepancy was only apparent, and, as far as Bengal was concerned, the disease was ' quite common '. He attributed the differences in hospital figures mainly to differences in the outlook of the medical personnel. He described the clinical features of his cases and observed that a large proportion of the cases which came to hospital were advanced cases ; that they occurred more commonly in men and ' more frequently in those persons of better class who are well educated and lead more or less a sedentary life '. In his opinion pyorrhœa alveolaris was not more common in these cases ; previous intestinal infections were not indicated as a causal factor and syphilis played no important role. He deprecated medical treatment for this condition amongst the hospital class of cases and advocated surgical treatment.

Nine years later, Somervell and Orr (1936) gave a fuller account of peptic ulcer in South India (observations based on 2,500 cases and nearly 600 followed-up cases). They stated that these cases of ulcers in South India showed some atypical characteristics, e.g., (*i*) rarity of perforation and hæmorrhage ; (*ii*) rarity of acute appendicitis but very marked association of chronic appendicular involvement—73 per cent of their cases showed tenderness in the appendicular region at the time of clinical examination ; (*iii*) prevalence of gastritis and duodenitis leading on to ulcer formation. In addition to these atypical features they found that in Travancore the distribution of peptic ulcer was not uniform : ' in the extreme south of the state it is comparatively rare ; on the sea coast it is less common than inland ; and in the northern and especially the central half of the country, it is very prevalent indeed '. Again, it was their clinical experience that gastric ulcer was relatively far more common in the Madras district than it was in Travancore where duodenal ulcers out-numbered the gastric variety by 30 : 1.

While discussing the various ætiological factors the authors laid great stress on the dietetic factor and stated that it was more than an accessory factor. Greater incidence of the disease was attributed to the habit of eating tapioca root. An interesting observation was made that whereas Tinnevelly district in South India used to be free from duodenal ulcer it had recently adopted the habit of eating tapioca root, and was now producing a continually increasing number of cases of this disease.

McCarrison (1912) recorded changes in the duodenum and upper intestine amongst guinea-pigs fed on a scorbutic diet of crushed oats and autoclaved milk. Four animals were used in this experiment. Death in all occurred within 46 days of the initiation of the experiment. He states, ' It appears then that congestive,

hæmorrhagic, atrophic and necrotic changes in the bowel walls, usually most pronounced in the upper intestine, are common consequences in guinea-pigs on dietaries deficient in accessory food factors '. This experiment was repeated by him with similar results. McCarrison (1931*a*, *b*) in his second experiment found ' duodenal ulceration at all stages of the process up to perforation (in one case) '.

Starting in 1927, McCarrison (*loc. cit.*) extended his observations and submitted the dietetic factor to test. Feeding experiments on albino rats with a diet similar to that in common use amongst the South Indians was undertaken. The animals were fed on Madras and Travancore diets for purposes of comparison and the laboratory stock on ' normal diet ' was used as controls. Scrutiny of his protocols showed that by feeding albino rats up to 675 days with the two types of diet in use, namely Madrasi and Travancore diets, no case of duodenal ulcer occurred and duodenitis was found in 3 animals out of 34, i.e., 8·82 per cent. In all these animals death occurred from an acute infection, i.e., enteritis in one (R 1509) and pneumonia with pleurisy in the other two (R 1517 and R 1531). About 20 per cent of the 34 animals showed lesions in the stomach which from the description appear to have been of an acute nature. Roughly 43 per cent of these animals died of pneumonia. Other less common causes of death were anæmia, enteritis and inanition. In view of the fact that acute ulcers in the stomach occur after acute infections both in experimental animals and in man, the above experimental evidence seems hardly sufficient to justify the conclusion that the dietetic factor played any prominent part in the ætiology of the lesions seen in the animals of the experiments under consideration. McCarrison (*loc. cit.*), however, concluded, ' Here, then, is unequivocal proof that two diets in use by certain people in India are capable of causing peptic ulcer in albino rats. The high incidence of this condition among the human users of these diets may, therefore, be definitely attributed to this cause. As to the mechanism of ulcer production—that is another matter ; the one that concerns me as an investigator in the service of the Indian people, is that two of the diets used by them do cause peptic ulcers, while a third, on which my stock rats are fed, does not. For them the moral is unmistakable '.

This work has not been confirmed. Orr and Rao (1939) working at Coonoor carried out feeding experiments and report that in no case were they able to produce ulcers of the stomach or duodenum in either albino rats or dogs fed on diets resembling those consumed by the poorer classes in the Madras Presidency and Travancore.

It must be pointed out that in man, here in South India, the problem is one of a chronic, slow-growing but progressive lesion in the first part of the duodenum and not acute ulcers of the stomach. In any experimental work there must, therefore, necessarily be reproduced the pathological process similar to what pertains in man. Again, disorders of the alimentary tract are the result of dysfunction of an extremely complicated alimentary system finely adjusted and dependent upon various factors, chemical, biochemical and neurological. Radiological studies of the alimentary tract, during recent years, have shown the extreme variability and adaptability of various parts of the tract to postural, physiological and emotional

responses. It would appear, therefore, that full cognizance must be taken of all these factors when experimental studies on this system are made and consequently the animal for experimentation should be one with an alimentary system, as much as possible, similar to that of man.

In view of the conflicting opinions on the clinical features of the disease, absence of any detailed studies in pathology of peptic ulcer in man as it occurs in South India and absence of any conclusive experimental evidence in favour of any particular ætiological factor or factors it was deemed necessary to prosecute further studies on this disease if one was to be able to indicate the factor or factors responsible for the high incidence, if at all, of the disease in South India. The present investigation was started early in 1938.

At the outset it was realized that a detailed study of the environment and habits of the sufferers from this condition would have to be made. The exact pathological lesion or lesions had to be studied and finally a correlation made of the pathological findings and the clinical picture in relation to environmental conditions before one could obtain a true perspective of the disease.

II. Materials and methods.

Thanks to the assistance of the staff of the Government General Hospital, Madras, opportunities were made available to me to study a large number of gastric cases. All the cases investigated were examined clinically, the history of illness inquired into and recorded; the fractional test-meal findings and radiological examination results, when available, were noted. Special pains were taken to inquire into the diet of the patient, laying special stress on the type of food eaten, the time of meals and ingredients of the diet. Inquiry was made with regard to habits of smoking, consumption of alcohol, etc., and particular attention was paid to inquiries as to the amount of the family income and the number of members of the family supported by the same. In all the cases studied the diagnosis had been confirmed at operation. The author attended all these operations and in each case noted site and size of the lesion, the amount of induration and pyloric obstruction present, enlargement or otherwise of the stomach and appearances of the gall-bladder. In most cases the appendix was removed and was thus available for histological examination. The cases were not selected in any way.

The present communication is an analysis of 258 consecutive proved cases of gastro-duodenal ulcers operated on by the first and second surgeons at the Government General Hospital, Madras. It may be noted here that all these cases were of a chronic nature. Acute perforating ulcers were usually emergency operations and were not attended by the author. In any case they were very few in number.

III. Incidence and distribution.

It is a general belief that peptic ulcer is far more common in the South than in the North of India : 58 times more common (McCarrison, 1921), while Somervell and Orr (*loc. cit.*) estimated it as 600 times more common.

Hingston (*loc. cit.*), Bradfield (1927), Somervell (*loc. cit.*), Somervell and Orr (*loc. cit.*) and Rao (1938) have attempted to show the prevalence of the disease from hospital statistics. Unless these statistics include all sources and data are especially collected for the purpose at the source, they cannot denote the correct extent of prevalence of the disease or give any indication with regard to its distribution.

The Map shows the distribution of the cases under review by districts. It will be seen that the number of cases decrease the farther we go from Madras. This, however, is obviously not a true picture. In districts where facilities are available and the people have confidence in the local medical authorities and their surgical talent, the cases are dealt with locally. In the present series of cases a vast majority had come from out-stations for treatment at the Government General Hospital, Madras. Of the 258 cases only 54 were residents of Madras proper. Similar conditions prevail at other centres.

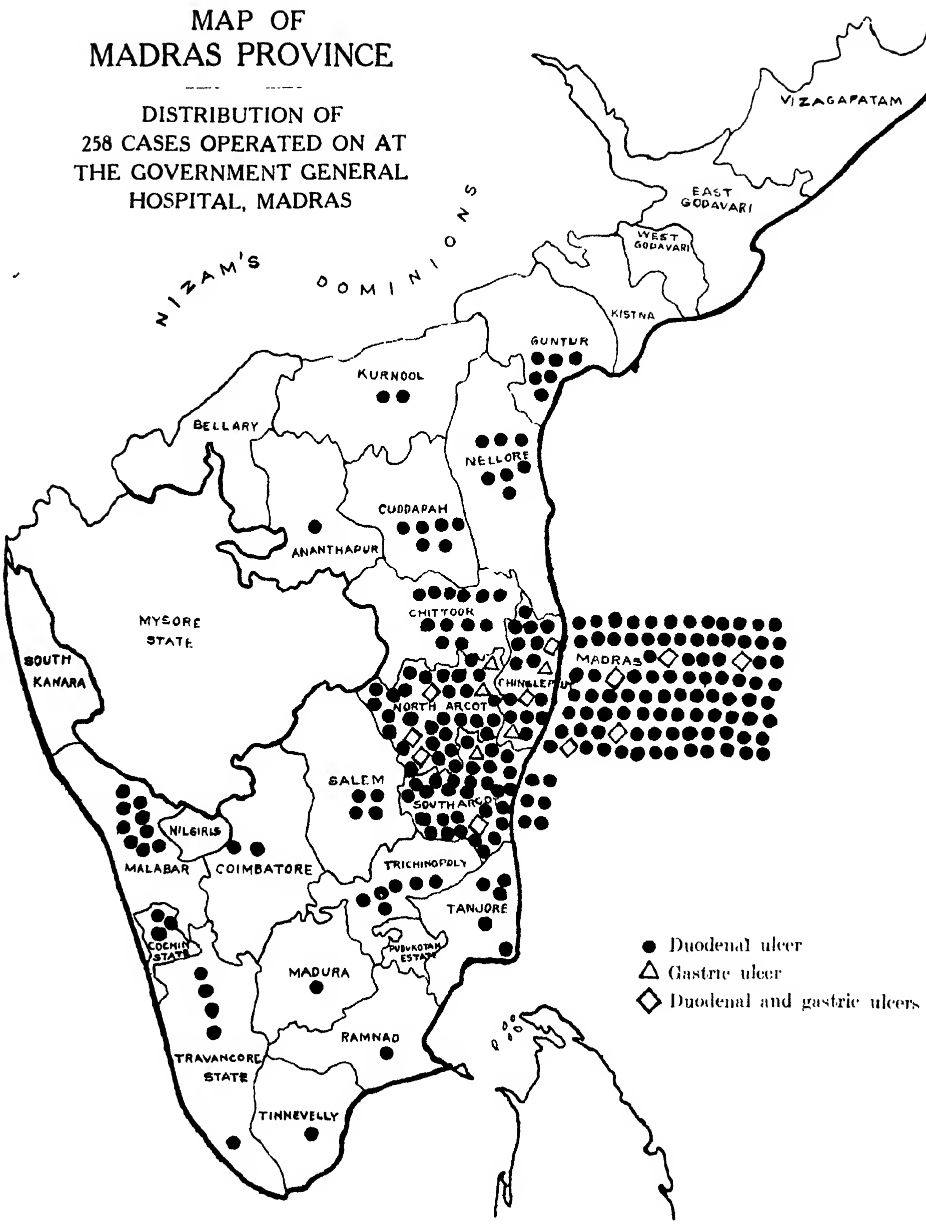
In the extreme south, London Mission Hospital, Neyyoor, and District Headquarters Hospital, Madura, deal with hundreds of cases annually drawn from the adjoining districts. King George's Hospital, Vizagapatam, similarly deals with cases in the northern parts of the province. Some other hospitals operate on a few advanced cases of the disease but are content to treat the rest medically until either presumably the patients die or go to the nearest big centre for operative treatment. The areas from which cases drain into the large centres mentioned above depend chiefly on geographical and economic reasons but the fame of the surgeon also plays a great part. The author has, therefore, been forced to enlist the co-operation of all the hospitals in the province and the neighbouring states to obtain data over a long period to ascertain the exact distribution of the disease and its incidence.

IV. Site of ulcers.

In the present series of 258 proved cases, the distribution of ulcers was as under :—

Duodenum only	241
Stomach only	5
Duodenum and stomach (pyloric and lesser curvature)	12

The relative proportion of gastric and duodenal ulcers hasbeen estimated and reported on by several workers. For purposes of comparison, statistics from autopsy records are excluded for obvious reasons. The mortality rate of the two conditions is disproportionate to the incidence of the two lesions, and, therefore, all that ends up in the mortuary is not directly proportional to what a surgeon sees in the operation theatre. Again, figures compiled from records of in-patients at a hospital are not necessarily reliable unless, as in the present series, the diagnosis has been proved at operation, or diagnosis is confirmed by careful radiological and biochemical investigations of the stomach and duodenum.


MAP OF
MADRAS PROVINCE
DISTRIBUTION OF
258 CASES OPERATED ON AT
THE GOVERNMENT GENERAL
HOSPITAL, MADRAS
NIZAM'S DOMINIONS
VIZAGAPATAM
EAST GODAVARI
WEST GODAVARI
KISTNA
GUNTUR
KURNOOL
BELLARY
NELLORE
CUDDAPAH
ANANTHAPUR
MYSORE STATE
SOUTH KANARA
CHITTOOR
MADRAS
CHINGLEPUT
NORTH ARCOT
SALEM
SOUTH ARCOT
NILGIRIS
MALABAR
COIMBATORE
TRICHINOPOLY
TANJORE
COCHIN STATE
PUDUKOTAH ESTATE
MADURA
RAMNAD
TRAVANCORE STATE
TINNEVELLY
Duodenal ulcer
Gastric ulcer
Duodenal and gastric ulcers

It is realized that the number of cases in the present series is very small, particularly for purposes of statistical evaluation, but examination of records of some hospitals in South India is in progress, for the purpose of reviewing a larger number of cases. The proportion of gastric and duodenal ulcers is, however, even in this series of a small number of cases, considered significant.

Analyses of cases operated on for this disease have been made by Moynihan (1920), Wilkie (1927), Young (1927), Mayo (1920), Soutter (1927) and others; in India, by Bradfield (1927), Somervell (*loc. cit.*), Somervell and Orr (*loc. cit.*), Rao (*loc. cit.*) and Kini (1939). These are given in Table I :—

TABLE I.

Incidence of gastric and duodenal ulcers in both sexes.

	Duodenal.	Duodenal and gastric.	Gastric.	Proportion of gastric to duodenal ulcers.
Moynihan (1920) (Leeds) ..	563	44	200	1 : 2½
Wilkie (1927) (Edinburgh) ..	310	51	52	1 : 6
Young (1927) (Glasgow) ..	244	..	30	1 : 8
Soutter (1927) (London) ..	615	..	660	1 : 1
Mayo (1920) (America) ..	4,532	203	1,171	1 : 4
Bradfield (1927) (Madras) ..	396	15	33	1 : 13
Somervell (1927) (Travancore) ..	514	4	14	1 : 32
Rao (1938) (Northern Circars) ..	466	14	71	1 : 6
Kini (1939) (Vizagapatam) ..	..	..	..	1 : 30·4
Present series (Madras) ..	240	12	5	1 : 48

The above figures indicate (*a*) the great discrepancy between the relative proportions of gastric and duodenal ulcers as observed in Great Britain and America on the one hand and South India on the other hand ; (*b*) that in South India duodenal ulcer greatly out-numbers the gastric variety, so much so that a typical lesser curvature ulcer of the stomach may almost be considered a rare condition ; (*c*) the conditions are similar whether you observe them in Vizagapatam which is in the north of the province, Madras and Madura in the central region, or Neyyoor in the extreme south of the Peninsula. The problem thus resolves itself into one of a duodenal ulcer, an ulcer that involves the first part of the duodenum either alone or associated with a similar ulcer in the pyloric antrum. Speaking generally, the type of lesions obtained on the East and West coast, the northern, central or extreme southern parts of the province is the same.

V. Clinical features.

Bradfield (1922), Pandalai (1923) and Somervell and Orr (*loc. cit.*) have described the clinical features of cases of this disease in South India. The last-mentioned writers, drawing attention to atypical features of their cases, stressed the striking rarity of perforation and hæmorrhages, the marked chronicity of the lesion associated with cicatrization and stenosis of the duodenum and tremendously dilated stomach.

A study of the present series of cases suggested a progressive pathological process which, at its various stages, showed different symptomatology but at each stage the symptoms were typical of the corresponding pathological lesion. For purposes of description the cases may be divided into two groups :—

(*i*) *The early cases.*—In the present series there were only 35 such cases. They conform to the typical textbook description of cases of duodenal ulcer, i.e., (1) classical hunger pains occurring regularly in relation to food, depending on the type of diet and quantity of food taken, but, for the same patient, occurring at the same time and relieved by taking food. The patients volunteered the statement regarding pain disturbing sleep at night ; vomiting was not a feature of these cases. Pain was of a gnawing character occurring in the epigastrium and usually slightly to the right of the middle line. (2) Radiological examination showed rapid emptying with tenderness and deformity of the duodenal cap. (3) Gastric analysis with the fractional test-meal technique showed the picture so well described by Ryle (1926) : 'the fasting juice is increased up to 80 c.c. to 100 c.c. or more ; it is perfectly clear, limpid and highly acid and contains little or no mucus. After the initial fall in acidity following withdrawal of the fasting specimens and dilution with gruel, there is a steady rise to a high point and thereafter either a steep drop with excessively rapid emptying or a sustained plateau with rapid or normal emptying and continuous after-secretion. In these cases there is evidence of a hyper-secretion and continued secretion which are seldom found in other dyspepsias'.

The patients were mostly young and not markedly emaciated but had the general state of under-nourishment characteristic of the hospital class of patients. At operation the stomach was normal in size and shape with no pyloric or duodenal obstruction. A small indurated ulcer on the anterior wall of the first part of the duodenum just beyond the pylorus was seen with no other changes in the viscera.

(*ii*) *The late cases.*—Eighty-six per cent of the cases under investigation may be grouped under this heading. All of them showed varying degrees of duodenal or pyloric stenosis resulting from cicatrization of a duodenal or a duodenal and pyloric ulcer. These cases showed absence of typical symptomatology of a pure duodenal ulcer.

(1) *Emaciation.*—Depending on the degree of stenosis patients showed varying degrees of emaciation with varying degrees of dilatation of the stomach. Bradfield (1924) drew attention to this fact when he observed 'the desperate condition in

which these patients have arrived before coming to the hospital is shown by the record of their weights. No less than 21 of the 50 patients under report weighed under 80 lb. while one weighed only 40 lb. and the other two 65 lb. and 64 lb., respectively'. The chronic starvation resulting from stenosis is often very marked, thus rendering patients extremely weak and debilitated and consequently bad subjects for operative procedures. In the present series weights were not recorded but emaciation and underweight was marked. Associated with this were varying degrees of anæmia and low blood pressure. Table II shows some typical examples of blood-pressure figures in such cases:—

TABLE II.

Blood-pressure reading taken before operation.

Duodenal ulcer (proved cases).

Duration of illness in years.	Age in years.	Blood pressure.		Case number.
		Systolic.	Diastolic.	
1	42	120	88	237
1.5	30	125	80	242
2	30	96	64	91
3	29	90	60	132
5	30	110	70	142
10	30	90	65	162
12	50	100	70	136
15	37	90	65	207
16	30	95	70	176
20	60	120	75	152

(2) *Pain and vomiting.*—The majority of cases complained of pain in relation to food, varying from pain soon after food to an hour or two after food. Pain was not localized to one spot in the epigastrium as in the early cases. In most cases pain was relieved by induced vomiting. Patients learn by experience that tickling the back of the throat with the forefingers is the easiest way of relieving pain. I have noted patients with marks on the back of the fingers produced by this practice. In cases of marked obstruction food ingested days before was seen in the vomitus. In places where facilities are not available for radiological examination to determine

obstruction the patient is given a meal of rice and kanji at night and the stomach washed next day when, in such cases, the previous night's meal can be recovered.

The mechanism of pain in these cases will be discussed in another communication. It may, however, be pointed out that there was severe recurring pain associated with increasing peristaltic vigour. At a later stage, however, pain was not a feature and the patients complained of a 'rolling in the stomach', due to futile efforts on the part of the stomach to propel its contents beyond the pylorus. This manifests itself in the form of visible gastric peristalsis which is easily demonstrated in such emaciated subjects by making them swallow a tumbler full of water and subsequently observing the phenomenon in the region of the stomach. Visible gastric peristalsis was observed in 165 cases of the total number of advanced cases, i.e., 75 per cent. This sign was not elicitable in any of the early cases described above.

(3) *Radiological examination.*—This was carried out only in a limited number of cases. In advanced cases the visible gastric peristalsis associated with profuse vomiting and other clinical features were sufficient for practical purposes of diagnosis and radiological examination was not done for reasons of economy and saving of time. Very occasionally the obstructive syndrome may be reproduced by the spasm associated with a non-obstructive lesion. Also it was often necessary to know the degree of obstruction when radiological examination was considered advantageous. Nothing unusual was found in the cases thus examined except the classical radiological findings already described in detail by various workers, Hurst and Stewart (1929) and Barclay (1910), etc.; suffice it to state that radiological investigations of the alimentary tract by the barium-meal technique is of considerable importance and assistance in early cases. Palpation and screening enable localization of areas of tenderness, filling defects of the duodenal cap, ulcer craters, etc. Radiological technique has advanced to a very high degree of accuracy and study of the gastric mucosa is of considerable help in diagnosis of the diseases of this organ.

(4) *Biochemical investigation.*—Only 35 per cent of the cases studied were investigated by the standard technique of fractional test-meal examination. It has already been pointed out that typical high acid curves were obtained in all uncomplicated early duodenal ulcer cases. In the advanced cases with varying degree of pyloric obstruction the test-meal results were neither reliable nor typical for the simple reason that a large number of such cases had associated gastritis with concomitant excess in mucous secretion, and, in some, had associated pyloric ulcers. One is inclined to agree with Hurst and Stewart (*loc. cit.*) when he states, 'Complete achlorhydria never occurs in duodenal ulcers'. He showed that in complicated cases of duodenal ulcer, such as are under consideration here, a second test-meal examination following a lavage gave higher acid values. Further work on this aspect of the question is in progress and will be communicated with a fuller discussion on the subject separately. Table III shows the relative frequency of different types of test-meal curves seen in duodenal ulcer cases. The arbitrary classification adopted is that advocated by Hurst and Stewart (*loc. cit.*).

TABLE III.

Relative frequency of different types of test-meal curves.

Duodenal ulcer (proved cases).

Case group.	Total.	Achlorhydria.	Hypochlorhydria.	Low-normal.	Average-normal.	High-normal.	Hyperchlorhydria.
Early cases without obstruction ..	20	*Nil*	*Nil*	1	2	2	15
Advanced cases with obstruction ..	69	1	1	12	13	16	26

It will be observed that amongst the early cases marked hyperchlorhydric and high-normal curves were present in 85 per cent of the 20 cases, with no case of achlorhydria or hypochlorhydria. On the other hand, amongst the late cases hyperchlorhydria and high-normal curves were obtained in only 61 per cent of the 69 cases studied. In 36 per cent average-normal and low-normal curves were seen and achlorhydria and hypochlorhydria occurred in 3 per cent of this group.

General features of peptic ulcer cases.

Both groups of cases described above have certain common features. These common features are of considerable ætiological significance and will be discussed fully separately but are included here to complete the description of the clinical picture of the disease.

Family history.—Although cases of gastric and duodenal ulcers have been described in which family history has been noted yet Hurst (1920), in England, was the first to draw attention to the frequency with which members of one family may suffer from one or the other type of ulceration in the stomach and duodenum. He ascribed this to a congenital predisposition of individuals in a family to the development of gastro-duodenal ulcers and named this as 'the gastric and duodenal ulcer diathesis'.

Forty-nine cases (19 per cent) or roughly 1 in every 5 cases gave a family history of the disease with one or more members of the family affected. In 1 case No. 87 patient's father and grandfather had suffered from the same complaint—all cured by operation. Somervell refers to a family of five brothers all of whom suffered from duodenal ulcers and were operated upon by him. This evidence, however, is not considered sufficient to support the diathesis theory for explaining this familial incidence. It may be stated that the joint-family system is an

outstanding feature of South Indian life and several generations of a family live under the same roof in exactly similar economic and social environmental conditions at one time. This environmental factor, perhaps, is more the cause of high familial incidence than any congenital predisposition.

Age and sex incidence.—The number of cases (245) under review is too small for purposes of statistical evaluation. Suffice it to state that the youngest was a female aged 12 and the oldest a male aged 65. Table IV shows the frequency of chronic peptic ulcers within different decades:—

TABLE IV.

Proved duodenal ulcers—Total 245.

Age group, years.	Male.	Female.	TOTALS.
0–9 ..	..	..	..
10–19 ..	8	1	9
20–29 ..	61	1	62
30–39 ..	80	2	82
40–49 ..	71	1	72
50–59 ..	16	1	17
60–69 ..	3	..	3
TOTALS ..	239	6	245

From this table it would appear that ulcers commonly occur between the ages of 20 and 40 years, the highest incidence being in the age group 30-39, both in males and females. The disparity of incidence between sexes is very great. In South India, Orr (1938) drew attention to this fact in his work in Travancore where he found 95 men to 5 women amongst cases of duodenal ulcer. The same was noted by other workers in South India. The reasons for this disparity in sex incidence are not known. This discrepancy can hardly be explained by the general biological sex differences usually noted in most diseases. Work on this aspect of the problem is in progress.

Religious groups.—Bradfield pointed out that incidence of ulcer according to religious groups conforms to the population group ratio. There are very wide differences in food, customs and environments of the different religious groups. Amongst the Hindus, again, the Brahmins are a distinct community differing very widely from the non-Brahmins and depressed classes, etc. This aspect was, therefore, further investigated with interesting results.

For this purpose 51 male cases of duodenal ulcer operated on at the Government General Hospital, Madras, were considered. They were all residents of Madras. Table V shows the total male population of different communities and the number of ulcer cases in each community in the 51 cases:—

TABLE V.

Religious groups : Peptic ulcer.

Religion.	Total male population.	Ulcer cases.	Ratio.
Brahmins ..	32,868	1	1 : 32,868
Non-Brahmins and depressed classes.	240,801	48	1 : 5,017
Mohammedans ..	38,341	2	1 : 19,170

It would appear that duodenal ulcer is more common amongst non-Brahmin Hindus than among Brahmins. Amongst the latter its occurrence may be considered very rare. This is further substantiated when it is considered that there were only two Brahmins amongst the 258 cases operated on in this series.

From a weekly census of patients of the Government General Hospital, Madras, over several weeks it was ascertained that various religious communities treated at the hospital were roughly in proportion to their population ratios. It may, therefore, be accepted that any difference in the incidence of the disease amongst the various religious groups is due to differences in the actual incidence of the disease in these groups.

Occupation, economic state and social conditions.—That the environment plays an important part in the ætiology of disease is now a concept beyond dispute. A detailed inquiry was made with regard to pertinent points and a brief account of results obtained is given here.

It has been pointed out that the joint-family system is an outstanding feature of family life in South India. Associated with this are early marriage, almost compulsory marriage for all and distribution of the entire income of the family amongst its members. Inquiry amongst 186 cases showed that 80 per cent were married, and with rare exceptions all had children. Amongst the 100 families

investigated in detail there were 277 children with an average of 2·7 per family. The average income of these families works out at 8 annas per day. In an average family of 3 children, husband and wife and perhaps a few dependents like a widow or an invalid, the total income available for food, clothing, housing, etc., is insignificant. Again, in larger families, e.g., case No. 200, labourer, although the daily income of the father was 12 annas a day supplemented with 8 annas by his eldest son, the total amount of 20 annas was all that was available for the husband, 2 wives and his 7 children. Case No. 226, ryot with a wife and 9 children had an income of 5 annas per day.

With regard to occupation, information was obtained from 243 patients of whom nearly 50 per cent were ryots and labourers. The remainder were weavers, petty merchants, servants, beggars and destitutes, etc.

The outstanding feature in this series is the extreme poverty of those affected. An occasional case may occur amongst the well-to-do (earning more than 16 annas a day) but it appears that, in South India, duodenal ulcer is a disease of the poor and consequently of the very low social stratum of the population. The rôle of this finding will be fully discussed elsewhere under ætiology of peptic ulcer in South India.

Diet.—From what has been said above it is apparent that not much of the family budget is available for purchase of food after paying for necessary expenditure on ceremonies connected with births, deaths, marriages, etc. The diet of these patients in general is meagre, unattractive and monotonous.

The majority of cases reported themselves as non-vegetarian (92·5 per cent). It may, however, be pointed out that although people would eat meat, fish and eggs when obtainable, it is very seldom that they get these articles and, for all practical purposes, most of the people may be considered as vegetarians, their food consisting of rice in most districts (chiefly Tamil) and ragi in some areas (mostly Telugu districts); tapioca is the staple food in Travancore but is not eaten, in any quantity, elsewhere.

In an average agriculturist family all the adults wake up before sunrise and proceed to work without any food. The first meal is cold usually taken at about 8 to 9 in the morning and consists of rice cakes or ragi left over from the night before, along with pickles or raw onions and salt. The next meal is at midday consisting of rice or ragi supplemented with 'kolambu' or 'rasam'; kolambu is a thick viscid stuff made by boiling tamarind, dhals, vegetables (radish or brinjals), salt, chillies and condiments. This concoction is boiled till it is thick. This procedure, incidentally, destroys all the valuable vitamins. No ghee or butter is used in the preparation. A small quantity of oil is often used for seasoning food preparations. Long after sunset, when all the work at the fields, including attending to cattle, is finished and the women have had time to cook food, the last and the principal meal of the day is eaten. This is a hot meal freshly prepared and consists of rice (boiled) with usually vegetable curry. No fresh fruits are eaten. Plantains are an occasional luxury, which are cultivated to be sold. Those that are not sold are dried and utilized for making curries. No milk or milk products form any part of the South Indian agriculturist's diet.

The children eat the same food from the age of two years onwards and field labourers who work on daily wages (2 to 4 annas per day) and do not own land cannot even afford the above. Their meals are even more scanty and consist chiefly of boiled rice or ragi supplemented by chillies and salt.

That this diet is unbalanced. below average requirements and lacks certain essentials by way of vitamins, fats, etc., is quite apparent. The rôle that diet plays, if any, in the ætiology of peptic ulcer in South India is being investigated and will be discussed elsewhere. Suffice it to mention here that although the meals are taken at regular times the interval between meals is very great and often the people must go about with empty stomachs for long periods, a fact which may have a considerable bearing on the causation of peptic ulcer.

Habits of smoking tobacco and drinking alcoholic beverages have been considered to play an important part in the ætiology of the disease. In the present series of 238 cases 64·7 per cent were smokers of indigenous cigarettes called 'beedies' and a few chewed tobacco. 38·7 per cent were addicted to alcohol. The quantity taken was small and at very varying intervals. Hardly anybody consumed alcohol daily. Only 25·2 per cent smoked tobacco and took alcohol as well.

VI. Summary.

1. Literature on peptic ulcer in South India has been reviewed showing divergence of opinions on various aspects of the disease and justification for the present investigation.

2. From a clinical study of 258 proved cases of peptic ulcer at the Government General Hospital, Madras, it has been shown that gastric ulcer of the lesser curvature of stomach is a rare disease and that so-called 'peptic ulcer' is mostly an ulcer of the anterior wall of the first part of the duodenum, a chronic lesion resulting in stenosis of the duodenum, associated in some cases with an ulcer in the pyloric antrum.

3. Eighty-six per cent of cases investigated were at the advanced stage of the disease associated with varying degrees of duodenal or pyloric obstruction and corresponding signs and symptoms of emaciation, low blood pressure, varying periods of pain in relation to food, vomiting and visible gastric peristalsis. Radiological findings and biochemical analyses in such cases are described.

4. A smaller number of early cases (14 per cent) has been described to show that there are no essential differences in the features of this disease in South India from those obtaining elsewhere.

5. High familial incidence is shown. The evidence available is not considered sufficient to support a diathesis theory but it is explained that a common environmental factor as obtaining under the joint-family system which is an outstanding feature of South Indian life is possibly the cause of a very high familiar incidence.

6. Great discrepancy in the sex incidence of the disease is shown, the age group chiefly affected being the same in both sexes, namely, 30–39.

7. The disease occurs amongst all religious groups but mostly affects the extremely poor and non-Brahmin Hindus. Amongst the Brahmins and the well-to-do it is extremely rare.

8. The diet of the agriculturist and labouring class of people is described and it is stressed that the interval between meals, frugal as they are, is very long. Whereas smoking is a common habit, drinking of alcoholic beverages is not so common.

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part IX.

QUANTITATIVE ASPECTS OF THE PROBLEM OF TOXICITY OF MUSTARD OIL.

BY

R. B. LAL, M.B., B.S., D.P.H., D.T.M. & H., D.B., F.N.I.,

S. P. MUKHERJI, M.SC.,

A. C. DAS GUPTA, L.M.F.,

AND

S. R. CHATTERJI, M.B., B.S., D.P.H., D.T.M.

(*An Inquiry under the Indian Research Fund Association.*)

(*From the All-India Institute of Hygiene and Public Health, Calcutta.*)

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Two physical and two chemical tests have been described in a previous communication (Lal, Mukherji, Roy and Sankaran, 1939) which could be employed to differentiate between samples of non-toxic mustard oil and toxic oil associated with epidemic dropsy. Evidence has also been presented to show that the toxic substance in the oil is derived from seed of *Argemone mexicana*, a weed found in varying proportions in many samples of mustard seed stocked for pressing oil. The causative rôle of this weed in epidemic dropsy has been supported by Chopra and his collaborators (1939). Our previous experience had suggested (Lal and Roy, 1939) that the severity of symptoms varied with the amount of mustard oil ingested and the degree of its toxicity which, in

turn, depended upon the concentration of argemone oil or to be more precise the amount of the toxic substance in the sample. Supplies of mustard seed from which oil is pressed were rarely if ever 100 per cent pure. As will be seen below a few argemone seeds sufficient to give faint reactions with the specific tests are frequently found in these stocks. It is, therefore, a matter of great practical importance to know whether it would be justified to discard all supplies of mustard oil in which slightest trace of the toxic substance could be demonstrated or was it possible to fix certain standards by which the edibility of the oil could be judged. To answer these questions certain issues have to be investigated :—

(*i*) To what extent seed of *Argemone mexicana* are actually found in stocks of mustard seed used for pressing oil.

(*ii*) Whether a quantitative test could be developed to detect the amount of toxic substance in terms of argemone oil in a sample of mustard oil.

(*iii*) What amount of toxic substance is required to be ingested to produce clinical symptoms.

I. Presence of argemone seed in stocks of mustard seed.

Some observations on this subject have been made in a previous communication (Lal *et al.*, 1939) where it has been shown that out of 40 samples of mustard seed, waiting to be crushed, in 40 different mills in and around Calcutta, 12 samples were found to contain ½ to 1 per cent and 5 samples 1 to 5 per cent argemone seed. In the remaining 23 samples no argemone seed could be detected. A point to note in this connection is that no separate records of the varieties of mustard seed in the samples were kept in that investigation. The significance of this observation will be appreciated from the following paragraphs. It may be mentioned here that these samples were collected at a time when epidemic dropsy was not widely prevalent in Calcutta and in the following 3 or 4 months no serious epidemics occurred.

Before describing further investigations on the extent to which argemone seed may be found in supplies of mustard seed an explanatory note on the varieties of these seeds is considered desirable. The name mustard seed covers a variety of oil seeds. They differ from each other both in size and in colour. *Brassica juncea* is a small-sized light-black seed commonly known as *rai* in vernacular. It closely resembles argemone seed in naked-eye appearance. *B. campestris* includes a number of varieties of brown (*torai, torial, kajli, toria,* or *lutni*) and light-yellow (*pilisarson*) seeds. The larger and lighter coloured seed of this species can be easily distinguished from argemone seed. *Gargar* (Bengal) and *gajra* (U. P.) are vernacular terms denoting a mixture of *rai*, yellow and brown seeds. To produce an oil of a particular colour, odour and pungency the different varieties of seed are mixed in fixed proportions before crushing. For a given brand of mustard oil the mixture has a definite composition and this is a closely-guarded trade secret.

Daily examination of mustard seed from an oil mill in Calcutta.

Daily samples of mustard seed of each variety* separately and of the mixture were obtained before crushing. Altogether 328 samples were received and examined. The results are shown in Table I-*a* :—

TABLE I-*a*.

Samples of mustard seed of different varieties arranged according to the amounts of argemone seed present. They were obtained daily from a certain oil mill in Calcutta.

Variety of seed.	PERCENTAGE OF *Argemone mexicana* SEED.						
	0	–1	–2	–3	–4	–5	5–
Pili sarson (*Brassica campestris*)	55	3	0	0	0	0	0
Brown *sarson* (*Brassica campestris*)	43	6	0	0	1	0	0
Rai sarson (*Brassica juncea*)	22	40	10	10	3	1	3
Gargar (mixture of *rai*, brown and yellow seeds)	5	16	2	2	0	0	0
Final mixture	25	70	7	3	1	0	0

In another series 7 samples were collected from stocks of mustard seed of wholesale dealers and 21 samples from different oil mills. The results are shown in Table 1-*b* :—

TABLE 1-*b*.

Samples of mustard seed of different varieties arranged according to the amounts of argemone seed present, obtained from certain mills and wholesale dealers.

Variety of seed.	PERCENTAGE OF *Argemone mexicana* SEED.							
	0	–1	–2	–3	–4	–5	–10	10–
Pilisarson (*Brassica campestris*) ..	5	0	0	0	0	0	0	0
Brown *sarson* (*Brassica campestris*) ..	0	0	0	0	0	0	2	1
Rai sarson (*Brassica juncea*)	0	3	1	0	0	0	7	0
Gargar (mixture of *rai*, brown and yellow seeds).	0	2	1	0	0	0	0	1
Final mixture	0	0	0	2	0	2	0	1

While a few stray grains of the weed may be found in all varieties of mustard seed it would be noted that for obvious reasons argemone seed is found with the greatest frequency and in largest amounts in stocks of *Brassica juncea* and in

* It is rare to find a sample containing exclusively one variety of seed, what is meant here is that one variety was the predominant type, the other varieties were found in small quantities only.

appreciable amounts in *gargar* and the final mixture. It might also be observed that, apart from the question of the variety of seed, the proportion in which argemone seed is found in stocks of mustard seed varies within wide limits. Indeed in some instances it is so high as to suggest deliberate adulteration. While the above observations suffice for our present purposes it would be a matter of considerable practical importance in connection with the control of the disease to obtain detailed information regarding the relative significance of various factors concerned in this variation; for instance it may possibly be found that seed from certain areas was worse than that from others or that the time of harvesting was a matter of importance in this connection.

II. Quantitative test for reacting substance in oil.

A general correspondence between the toxicity and intensity of reaction with nitric acid was demonstrated in Table II of Part VIII of this series (Lal *et al.*, 1939). This subject will be more fully discussed later on. Leaving aside, for the moment, the question of the identity or otherwise of the reacting substance with the toxic substance and, in case they are not identical, of the occurrence of the two substances in a fixed proportion in all samples of toxic oil a quantitative colorimetric test to determine the reacting substance content of a given sample of oil can be developed.

Technique.

Five c.c. each of the oil to be tested and of concentrated nitric acid (Merck's analytical reagent) are vigorously shaken in a test-tube for 2 minutes. The mixture is transferred to a centrifuge tube and centrifuged for 3 minutes at 5,000 revolutions per minute. The bottom acid layer is pipetted out and put into an absorption cell of suitable thickness. The colorimetric reading is taken by means of a Pulfrich photometer (Carl Zeiss Zena gradation photometer). Concentrated nitric acid is used as control. It is put in an absorption cell of equal thickness. In case of mixtures containing high concentrations of the reacting substance in oil the quantity of nitric acid is correspondingly increased.

The extinction coefficient (K) of a solution varies inversely as the thickness of the absorption cell and directly as the -log of penetrability expressed in DS, thus

$$K = \frac{\log DS}{S}$$

where S = thickness of cell in cm.

Since, however, the scale on the drumhead of the Pulfrich photometer directly gives the value of -log DS, the value of K can be obtained by dividing the reading on the drumhead by S.

There are four experimental conditions to be standardized to obtain comparative results. These are :—

(*a*) Wave-length, i.e., choice of filter.

(*b*) Time limit within which the experiment should be finished.

(*c*) Thickness of absorption cell.

(*d*) Dilution, i.e., proportion between the amount of oil and nitric acid.

(*a*) *Selection of wave-length at which the experiment is to be performed.*—To select the most suitable filter a series of experiments were carried out using three concentrations of argemone oil in jail produced pure mustard oil (to be called pure mustard oil for short) which was found negative to nitric acid test. The results are given in Table II :—

TABLE II.

Values of extinction coefficient of three different mixtures of argemone oil in pure mustard oil at different wave-lengths.

Concentration of argemone oil in pure mustard oil, per cent.	Dilution oil: nitric acid.	Thickness of absorption cell in mm.	Extinction coefficient using various filters or different wave-lengths.								
			750 M. U.	729 M. U.	680 M. U.	620 M. U.	572 M. U.	530 M. U.	494 M. U.	460 M. U.	434 M. U.
1	1 : 1	10·05	0·01	0·01	0·02	0·02	0·06	0·30	0·70	1·00	Hazy.
5	1 : 2	5·01	0·04	0·04	0·08	0·12	0·28	1·28	3·20	4·80	,,
10	1 : 2	2·47	0·08	0·08	0·24	0·24	0·64	2·40	7·20	10·24	,,

In order that the slightest variations may be easily appreciated it is necessary to select the wave-length at which maximum values of the extinction coefficient are obtained. The above table shows that the choice must rest on the wave-length of 460 M. U.

(*b*) *Time limit within which the experiment is to be completed.*—It was found that the extinction coefficient varied if the readings were taken at different periods since the commencement of the test. The standard period to be selected must necessarily be one within which the experiment could be performed with ease. However, if this period was unnecessarily long the values of extinction coefficient would progressively diminish (*see* Table III) and, therefore, shortest practicable time had to be taken as standard. This was found to be 20 minutes.

TABLE III.

Extinction coefficient of a 5 per cent mixture of argemone oil in pure mustard oil at varying periods.

Time in minutes.	Extinction coefficients at different wave-lengths.			
	572 M. U.	530 M. U.	494 M. U.	460 M. U.
20	0·24	1·36	3·36	5·00
40	0·24	1·20	3·20	4·80
60	0·24	1·20	3·20	4·40
180	0·24	1·12	2·72	3·84

Incidentally this table further supports the choice of wave-length at 460 M. U.

(c) *Thickness of absorption cell, and (d) Dilution.*—Provided there is enough nitric acid to complete the reaction any additional acid merely acts as diluent and reduces the value of extinction coefficient proportionately. The values of extinction coefficient given here relate to the equivalent of 1 : 1 mixture of oil and acid. If the colour is too strong perfect matching becomes difficult. There are two alternative remedies to overcome this difficulty, namely (1) the mixture should be further diluted with nitric acid, or (2) the thickness of the cell should be reduced. The manufacturers of the instrument recommend the use of thick cell for getting accurate results. However, as will be seen from Table IV, for all practical purposes there is no appreciable difference in the results between the values of extinction coefficient obtained with 10·05 mm. and 5·01 mm. thick cells. We have, therefore, used 10·05 mm. thick cell so long as distinct reading could be obtained with reasonable dilution. In case of very high concentrations of the reacting substance in the oil 5·01 mm. cell has been employed.

TABLE IV.

Relationship between the dilution and size of cell using 5 per cent mixture of argemone oil in pure mustard oil.

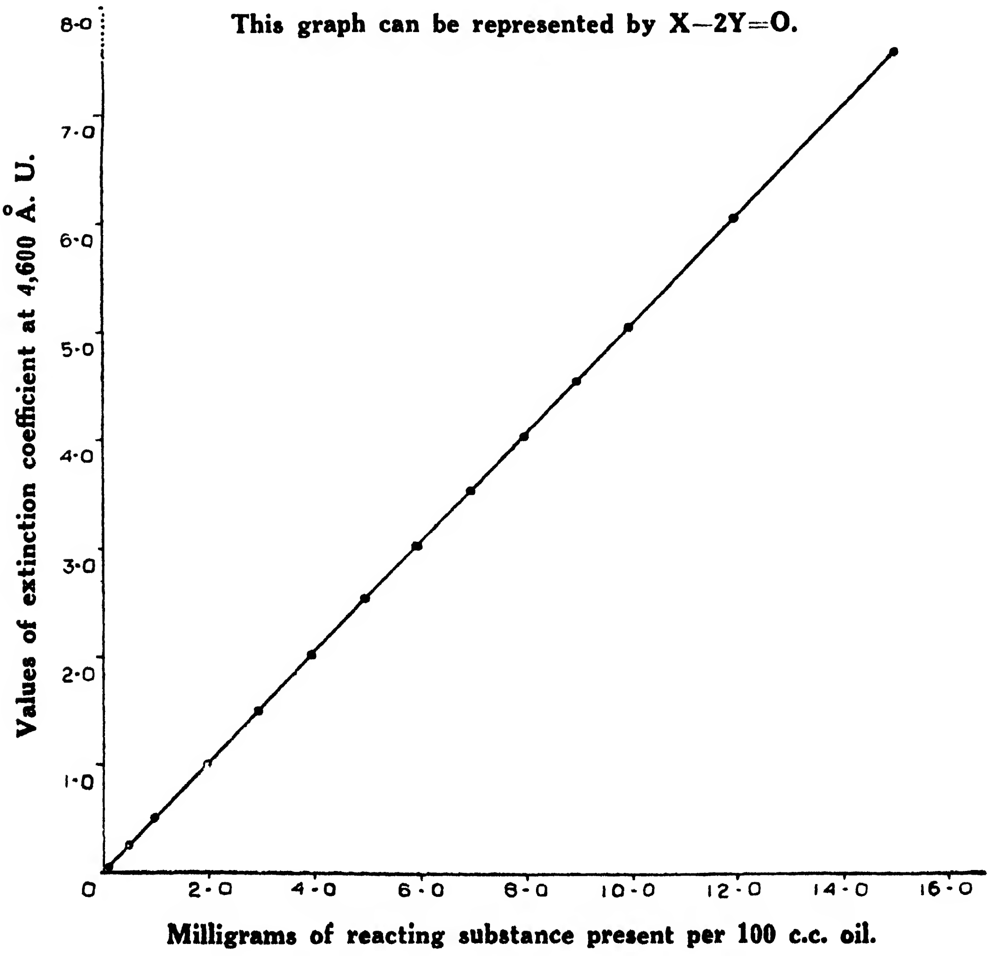
Thickness of absorption cell in mm.	Dilution. Oil: nitric acid.	Extinction coefficient at 460 M. U.
10·05 ..	1 : 1	Indistinct.
	1 : 2	4·60
	1 : 4	4·80
	1 : 8	4·90
5·01 ..	1 : 1	4·80
	1 : 2	4·80
	1 : 4	5·04
	1 : 8	4·90
2·47 ..	1 : 1	4·80
	1 : 2	5·04
	1 : 4	4·90
	1 : 8	4·90

Estimation of the amount of reacting substance in a given sample of mustard oil.

In order to facilitate the estimation of the reacting substance and roughly the quantity of argemone oil in a sample of mustard oil standard gauging curves have been worked out (*vide* Graphs 1 and 2). Having obtained the value of the extinction coefficient of the sample of oil to be tested, the quantities of the reacting substance (in mg. per 100 c.c.) and of the argemone oil (in c.c. per 100 c.c.) can be read off directly from Graphs 1 and 2, respectively.

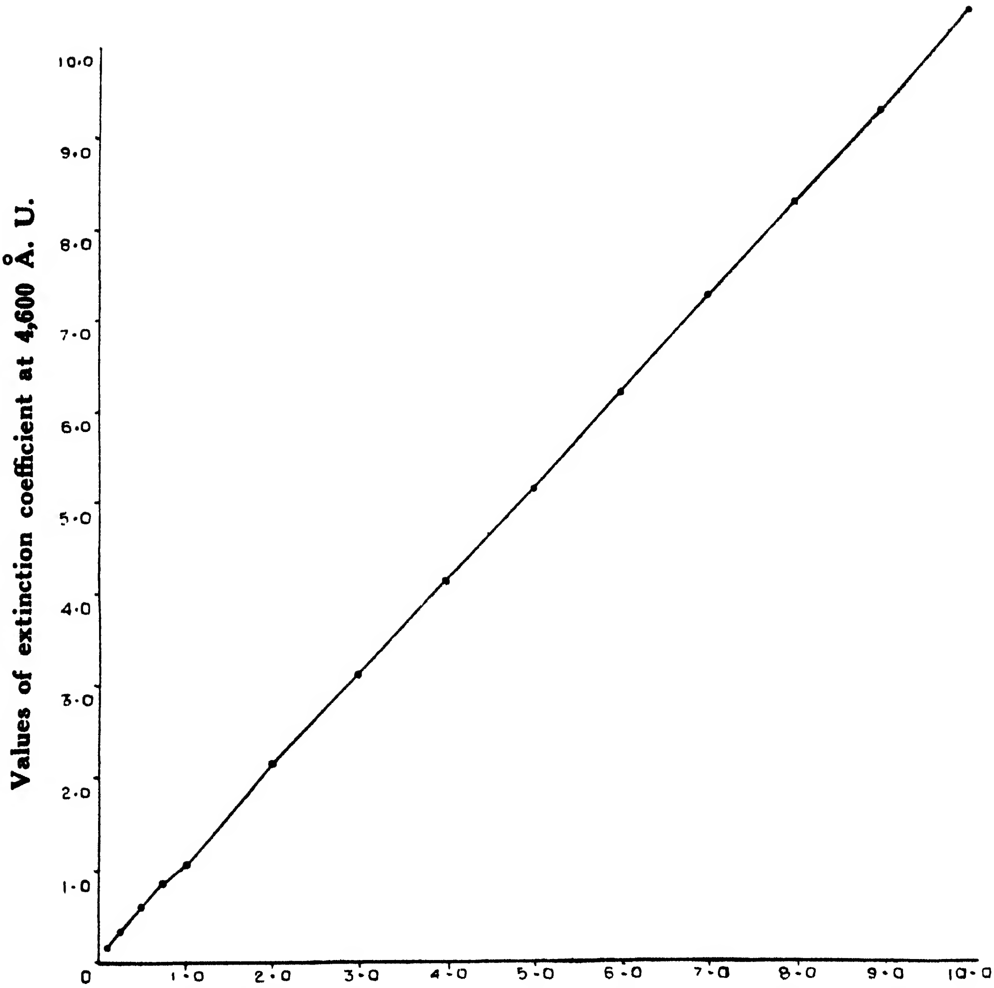
GRAPH 1.

Concentration of reacting substance and value of extinction coefficient of samples of oil.



GRAPH 2.

Concentration of argemone oil and value of extinction coefficient of samples of mixtures with mustard oil.



Percentage by volume of oil of *Argemone mexicana* in pure mustard oil.

Stability of reacting substance in oil.

An appreciable reduction in the values of reacting substance was noted in the same sample of oil on re-examination after an interval of about a month. This observation gave rise to doubts as to the value of the quantitative test described

above. The value of the test would naturally depend upon the identity or correspondence between the toxic substance and the reacting substance content of the oil. If as a result of storage or of other circumstances a reduction took place in the reacting substance only without a corresponding reduction in its toxicity the quantitative test would give spurious results with regard to toxicity. If on the other hand there was a parallel reduction in the two substances or in the two attributes of the oil, viz., reactivity and toxicity, the quantitative test would give a correct indication of toxicity. Leaving aside for the moment the consideration of the nature of relationship between toxicity and reactivity it was necessary to investigate into the factors concerned in causing reduction in the reactivity of the oil.

Repeated examinations of fresh mixtures of argemone oil in pure mustard oil showed that the degree of variation in results due to personal factor or variation in experimental conditions was negligible and the method was, therefore, reliable (*see* Table V).

TABLE V.

Extinction coefficient of fresh samples of various mixtures of argemone oil in pure mustard oil on repeated examinations.

Percentage of argemone oil in pure mustard oil.	Values of extinction coefficient at 460 M. U.
1·0 ..	(1) 1·00
	(2) 1·10
	(3) 1·00
	(4) 1·00
2·5 ..	(1) 2·60
	(2) 2·50
	(3) 2·60
	(4) 2·60
5·0 ..	(1) 5·10
	(2) 5·20
	(3) 5·20
	(4) 5·10

The difference in the values of extinction coefficient observed previously could only be ascribed to storage. To make sure that it was so a series of experiments with different mixtures of argemone oil in mustard oil were carried out—the samples were put in test-tubes and left on the laboratory table exposed to light. The results, average of 4 readings, are shown in Table VI :—

TABLE VI.

Reduction in the values of extinction coefficient of various mixtures of argemone oil in pure mustard oil after storage for a fortnight.

Percentage of argemone oil in pure mustard oil.	Extinction coefficient (estimated with fresh mixtures) at 460 M. U.	Extinction coefficient of the same mixtures after 15 days at 460 M. U. The samples being kept exposed to diffused light.	Difference between the two readings.
0·10	0·16	0·15	0·01
0·25	0·34	0·30	0·04
0·50	0·61	0·55	0·06
0·75	0·87	0·82	0·05
1·00	1·05	0·93	0·08
2·00	2·12	1·87	0·25
3·00	3·12	2·70	0·52
4·00	4·12	3·30	0·82
5·00	5·13	4·20	0·93
6·00	6·17	5·02	1·15
7·00	7·23	5·97	1·26
8·00	8·26	6·70	1·56
9·00	9·29	7·35	1·94
10·00	10·33	8·17	2·16

It will be observed that the reduction in extinction coefficient is consistent throughout.

The experiment described above was repeated with certain samples of toxic mustard oil obtained from the field. These samples had been stored in original phials in a dark place. The samples were taken out and their extinction coefficient was determined. They were then left exposed to diffused light on the laboratory

bench. The values of extinction coefficient of these samples were re-determined after 3 months. The results are set out in Table VII :—

TABLE VII.

Reduction in the values of extinction coefficient of certain samples of epidemiologically incriminated mustard oil after exposure to diffused sunlight.

Sample number.	Values of extinction coefficient at 460 M. U.		Deviation.
	First estimation.	Estimation after a period of 3 months.	
19	1·33	1·20	0·13
294	2·18	1·82	0·36
297	2·60	2·06	0·54
132	2·65	2·22	0·43
288	2·76	2·20	0·56
96	2·86	2·32	0·54
20	4·10	2·70	1·40
269	4·40	3·08	1·32
37	4·84	3·80	1·04

It will be observed that in all instances there was reduction in the extinction coefficient of roughly the same order as in the mixtures. This is an additional evidence in favour of the hypothesis that the source of the reacting substance (toxic ?) is argemone oil.

In order to further investigate the rôle of light in the process resulting in reduction of the reacting substance a series of experiments were performed in which direct instead of diffused sunlight was employed.

Effects of exposure to direct sunlight on pure argemone oil and on mixtures of argemone oil in mustard oil.

Pure argemone oil contained in glass-beaker covered with glass was exposed to direct sunlight for 5 hours and also for 36 hours (3 or 4 hours daily). The samples so treated were mixed with pure mustard oil to make 5 per cent mixtures. The experiments were repeated with this difference that the mixture in this case was made before and not after the exposure. Freshly made unexposed mixtures of the same strengths were used as controls. In the mixtures, exposure for longer periods than 5 hours gave results which were difficult to interpret because after 10 hours' exposure the extinction coefficient at 494 M. U. was higher than at 460 M. U. This was probably due to a distinct change which took place in the colour of the oil and consequently in the colour

of the acid layer which instead of being yellow or brown was of a pinkish tint. The results (average of 3 readings) are given in Table VIII :—

TABLE VIII.

Reduction in the values of extinction coefficient in the mixtures of argemone oil in pure mustard oil and pure mustard oil as such used as control after exposure to direct sunlight.

Oil exposed.	Values of extinction coefficient at 460 M. U.		
	Period of exposure in hours.	Control reading.	Final reading.
Pure argemone oil (5 per cent mixture of this treated oil was used for finding out its extinction coefficient).	5	4·90	2·90
	36	4·90	0·48
Five per cent mixture of argemone oil in pure mustard oil.	5	4·90	1·68
Pure mustard oil	5	0	0

Exposure to direct sunlight also caused reduction in the extinction coefficient values of the epidemiologically incriminated oils (*vide* Table IX).

TABLE IX.

Reduction in the values of extinction coefficient in samples of epidemiologically incriminated mustard oil caused by direct sunlight.

Sample number.	Values of extinction coefficient at 460 M. U.		Deviation.
	Before exposure.	After exposure to direct sunlight for 6 hours.	
19	1·20	0·64	0·56
294	1·82	0·58	1·24
297	2·06	0·52	1·54
132	2·22	0·68	1·54
288	2·20	0·68	1·52
96	2·32	0·96	1·36
20	2·70	1·20	1·50
269	3·08	0·92	2·16
37	3·80	1·28	2·52

The reacting substance which has been isolated in pure crystalline form from argemone oil was also exposed to direct sunlight. Similar treatment was given to a solution of the substance in pure mustard oil. The results are shown in Table X:—

TABLE X.

Effect of exposure to direct sunlight (in the presence of air) on the values of extinction coefficient of pure crystals of the reacting substance and of the solution of the substance in pure mustard oil.

Substance exposed.	Solution examined.	Period of exposure in hours.	Values of extinction coefficient at 460 M. U.		Deviation.
			Before exposure.	After exposure.	
Pure crystals ..	10·0 mg. dissolved in 100 c.c. HNO_3.	40	5·00	4·62	0·38
Mixture of pure crystals in mustard oil.	..	5	10·00	9·60	0·40
		40	10·00	2·20	7·80

The results of these experiments (Tables VI, VII, VIII, IX and X) may be summarized as follows:—

1. Light causes reduction in the values of extinction coefficient of samples of oil containing the reacting substance.
2. This effect is much more marked when the oil is exposed to direct sunlight than when it is exposed to diffused light.
3. Light has no effect on the reacting substance isolated in crystalline form except a change in colour after prolonged exposure. However, when re-dissolved in oil it is affected by light in the usual manner.

With a view to appreciating the nature of the reaction which results in reduction of the extinction coefficient value, certain circumstances influencing the reaction were studied.

1. *Ultra-violet radiation.*—In the experiments described above the containers in which oil was exposed were covered with glass tops. The observed reductions in the value of extinction coefficient were therefore due to visible part of the spectrum. In order to see whether the more chemically active part of the spectrum, viz., ultra-violet radiation, would act more vigorously, some experiments were carried out under

a mercury vapour lamp. (The British Hanovia Quartz Lamp, 450 watts and 220 volts). (*See* Table XI.)

TABLE XI.

Reduction in the values of extinction coefficient on exposure of a 5 per cent mixture of argemone oil in pure mustard oil in contact with air to visible and ultra-violet radiations.

Oil exposed.	Control reading.	Period of exposure in minutes.	Values of extinction coefficient at 460 M. U. — Ultra-violet radiation.	Values of extinction coefficient at 460 M. U. — Visible radiation.
5 per cent mixture of argemone oil in pure mustard oil.	4·90	10	4·72	4·60
		30	4·20	4·00
		60	3·40	3·10
		90	2·72	2·50
		120	2·28	1·90
		150	1·88	1·70
		180	1·84	1·64
		210	1·84	1·50
		300	..	1·60

It would thus appear that the reaction is brought about equally by visible and ultra-violet radiation.

2. *Heat.*—Since sunlight is accompanied by heat radiations, the effect of heat on the reacting substance was separately determined. The oil to be examined was heated at 250°C. for ten minutes. The results are shown in Table XII :—

TABLE XII.

Effect of heat on the value of extinction coefficient of pure argemone oil and its 5 per cent mixture in pure mustard oil.

Oil.	Values of extinction coefficient at 460 M. U. — Before heating.	Values of extinction coefficient at 460 M. U. — After heating.
Pure mustard oil	0	0
Mixture of 5 per cent argemone oil in pure mustard oil.	4·90	4·90
Pure argemone oil (values of extinction co-efficient determined with a 5 per cent mixture of this oil in pure mustard oil).	4·90	5·00

Apparently heat does not influence the reaction. This observation is of interest in connection with the finding of Chopra and his co-workers (*loc. cit.*), namely, that argemone oil heated to 240°C. loses its toxicity to laboratory animals. However, the criterion of toxicity used by them is not clearly mentioned.

3. The next question investigated was whether heat enhances the effects of light. For this purpose heated and unheated samples of argemone oil and a 5 per cent mixture of argemone oil in mustard oil were exposed to direct sunlight. The results are given in Table XIII :—

TABLE XIII.

Reduction in the values of extinction coefficient on exposure to light of heated and unheated samples of argemone oil and 5 per cent mixture of argemone oil in mustard oil.

Oil exposed.	VALUES OF EXTINCTION COEFFICIENT AT 460 M. U.			
	UNHEATED.		HEATED.	
	Before exposure.	After exposure.	Before exposure.	After exposure.
Pure mustard oil	0	0	0	0
5 per cent mixture of argemone oil in pure mustard oil.	4·90	1·68	4·90	1·54
Pure argemone oil (diluted after treatment to form a 5 per cent mixture with pure mustard oil).	4·90	2·90	5·00	2·70

These results require no further comments.

4. *Effect of exposure to light in the presence o air.*—In the previous experiments the samples were exposed to light in the presence of air. If at the time of exposure contact with air is prevented by filling the stoppered vessel up to the brim, the value of extinction coefficient is not reduced in the case of pure argemone oil while in the mixture reduction in extinction coefficient value takes place though less rapidly than in the presence of air. The results of experiments are given in Table XIV.

TABLE XIV.

Effect of light on the value of extinction coefficient of pure argemone oil and of a 5 per cent mixture of argemone oil in pure mustard oil **when air is excluded.**

Sample of oil.	Period of exposure.	Type of exposure. Direct or diffused sunlight.	Values of extinction coefficient at 460 M. U. Control.	Final.
Pure mustard oil	5 hours	Direct	0	0
	3 months	Diffused	0	0
5 per cent mixture of argemone oil in pure mustard oil.	5 hours	Direct	4·80	3·40
	3 months	Diffused	4·90	2·30
Pure argemone oil (diluted after treatment to form a 5 per cent mixture in pure mustard oil).	5 hours	Direct	4·90	5·00
	3 months	Diffused	4·90	4·80

However, as may be seen from Table XV the direct cause of reduction in extinction coefficient is light, in the absence of which contact with air does not induce any change. This is important because in actual practice, the oil is stored in tins which protect it from light although air may not be wholly excluded. We would, therefore, expect that under such conditions of storage there is no reduction in the reactivity of the oil. However, when mustard oil containing argemone oil is exposed to light and air during retail sale some reduction in its reactivity to the test must necessarily take place.

TABLE XV.

Effect of air on the extinction coefficient value of pure argemone oil and mixture of argemone oil in pure mustard oil **in the absence of light.**

Sample of oil.	Time interval between the control and final readings in months.	Air present or absent.	Values of extinction coefficient at 460 M. U. Control.	Final.
Pure mustard oil	3	Present	0	0
	3	Absent	0	0
5 per cent mixture of argemone oil in pure mustard oil.	3	Present	4·90	4·70
	3	Absent	4·80	4·60
Pure argemone oil (diluted after treatment to form a 5 per cent mixture of it with pure mustard oil).	3	Present	4·92	4·76
	3	Absent	4·92	4·70

It would thus appear that the reacting substance in the form in which it exists in argemone oil is photo-sensitive. It is destroyed by both direct and diffused light and equally by the visible and the ultra-violet radiations. It would be interesting to speculate as to the nature of the reaction. We have seen that air is necessary to affect reduction in the reacting substance content of pure argemone oil. This observation suggests that an oxidation process of photo-chemical nature is involved. How mustard oil supplies the necessary oxygen is not quite clear. In the presence of light the reacting substance would appear to possess a much greater reducing property than allylisothiocyanate which is present in the mustard oil without being able to react. In this connection our earlier observation on the comparatively greater reducing properties of toxic oil over non-toxic oil (Lal *et al.*, 1939) may be recalled. That no specific ferment of mustard oil is concerned in the process is evident from the fact that heating of mustard oil before mixing it with argemone oil does not interfere with the phenomenon. However, this does not rule out the possibility of a heat-stabile catalyst being involved in the process but this is very unlikely.

Another interesting fact to which attention may be directed is that while pure dry crystals of the reacting substance may stand considerable exposure to direct sunlight and air without loss of reactivity, they are unable to do so when dissolved in fats of mustard oil or argemone oil. This clearly suggests that the form in which the reacting substance exists in the oil is not identical with the pure crystalline substance. In this difference may possibly be found the key to the nature of the toxic substance.

III. Estimation of the quantity of toxic oil required for producing symptoms of epidemic dropsy.

Essentially the problem is to estimate the minimum amount of the specific toxic substance in the oil the consumption of which would on an average cause symptoms of the disease. There are two other issues involved with this problem, namely, whether (1) the rate of consumption of the toxic oil, and (2) the presence of any other item of food or any form of food deficiency enter as factors in the production of the symptoms or in determining their severity. The only way to solve the problem satisfactorily would be to carry out a large scale human feeding experiment with the toxic substance isolated in the pure form. This is not possible to do for two reasons: firstly, because the toxic substance has not been isolated and, secondly, because it is not possible to obtain sufficient number of human volunteers for this purpose. Only an indirect approach can therefore be made. Since, however, there must necessarily be provided a fairly large margin of safety in the permissible limit of the toxic substance in the oil, even an approximate approach to the quantitative aspect of the problem will be of practical value.

If it could be assumed, as appears likely*, that the toxic substance in the oil corresponds to its extinction coefficient value as determined by the quantitative

* Evidence on which this statement is based will be presented in a subsequent communication.

test described above, it may be possible to arrive at an estimate of the minimum toxic limit by exploring some of the available data in the following manners :—

1. Comparison of the extinction coefficient values of samples of oil with the records of toxic effects on the consumers, i.e., the number of persons affected. In estimating the toxicity of the oil the severity of the symptoms and the amount of the oil consumed and the period during which it was taken have to be taken into consideration.

2. Following oils of known extinction coefficient value to the consumers and noting the results or keeping a community under observation and carrying out concurrent examination of the oil consumed.

3. Experimental administration of oils of known extinction coefficient values.

1. *Comparison of toxic effects with extinction coefficient values of the samples.*

The quantitative test was developed at a late stage of investigations and by that time most of the samples of oil obtained from the field had become old. Moreover, many of them were collected long after the oil of which they were samples had been consumed. Since the extinction coefficient value of oil undergoes progressive diminution with exposure to light and air and the exact conditions of storage of the samples have not been recorded they may not be considered quite suitable for the present investigations. However, the available data are set out in Table XVI and a summary of the findings is given in Table XVII :—

TABLE XVI.

Comparison of toxic effects of certain oil supplies with the extinction coefficient of respective samples.

Sample number.	Toxicity as roughly judged from epidemiological history.	Extinction coefficient at 460 M. U.	Milligrams of reacting substance per 100 c.c. oil.	Approximate percentage of argemone oil.	Time interval in months between commencement of use of oil and date of quantitative examination of oil.	REMARKS.
39*	+++	5·00	10·00	4·88	8	
37*	+++	4·84	9·68	4·74	8	
20*	+++	4·10	8·20	4·00	13	
132*	+++	2·65	5·30	2·53	7	

* *Vide* Lal *et al.* (1939) for detailed information regarding the source, etc., of these samples.

TABLE XVI—*contd.*

Sample number.	Toxicity as roughly judged from epidemiological history.	Extinction coefficient at 460 M. U.	Milligrams of reacting substance per 100 c.c. oil.	Approximate percentage of argemone oil.	Time interval in months between commencement of use of oil and date of quantitative examination of oil.	REMARKS.
152*	+++	2·60	5·20	2·50	7	
297*	+++	2·60	5·20	2·50	8	
294*	+++	2·18	4·36	2·08	7	
15*	+++	1·92	3·84	1·80	14	
118*	+++	1·90	3·80	1·78	6	
167*	+++	1·68	3·36	1·57	6	
308*	+++	1·56	3·12	1·48	5	
301*	+++	0·01	0·02	0·01	6	
288*	++	2·76	5·52	2·64	9	
97*	++	1·95	3·90	1·84	7	
135*	++	1·63	3·26	1·53	..	
309*	++	1·36	2·72	1·30	5	
299*	++	1·24	2·48	1·19	7	
69*	++	0·04	0·08	0·03	9	
269*	+	4·40	8·80	4·30	7	
96*	+	2·86	5·72	2·74	7	
19*	+	1·33	2·66	1·27	13	
94*	+	1·10	2·20	1·06	7	
3*	—	0·90	1·80	0·80	14	
13*	—	0·18	0·36	0·12	14	
40*	—	0·04	0·08	0·03	..	
298*	—	0·03	0·06	0·02	..	

* *Vide* Lal *et al.* (1939) for detailed information regarding the source, etc., of these samples.

TABLE XVI—*concld.*

Sample number.	Toxicity as roughly judged from epidemiological history.	Extinction coefficient at 460 M. U.	Milligrams of reacting substance per 100 c.c. oil.	Approximate percentage of argemone oil.	Time interval in months between commencement of use of oil and date of quantitative examination of oil.	REMARKS.
221*	—	0·02	0·04	0·02	21	
55 (13)†	—	0·02	0·04	0·02	21	
52 (10)†	—	0·02	0·04	0·02	21	
54 (12)†	..	0·01	0·02	0·01	21	
50 (8)†	—	0·01	0·02	0·01	22	
220*	—	0·01	0·02	0·01	..	Jail produced mustard oil.
43 (1)†	—	0·01	0·02	0·01	22	
46 (4)†	—	0·01	0·02	0·01	22	
51 (9)†	—	0·01	0·02	0·01	21	
57*	—	0·01	0·02	0·01	21	
14*	—	0·01	0·02	0·01	15	
21*	—	0·0	0·0	0·0	11	
22*	—	0·0	0·0	0·0	11	
45 (3)†	·	0·0	0·0	0·0	22	
47 (5)†	—	0·0	0·0	0·0	22	
48 (6)†	—	0·0	0·0	0·0	21	
53 (11)†	—	0·0	0·0	0·0	20	
56 (14)†	—	0·0	0·0	0·0	21	
155*	..	..	..	..	..	Jail produced mustard oil.

* *Vide* Lal *et al.* (1939) for detailed information regarding the source, etc., of these samples.

† *Vide* Lal, Ahmad and Roy (1938) for detailed information regarding the source, etc., of these samples.

TABLE XVII.

Summary of data given in Table XVI.

Degrees of toxicity.	Amount of the reacting substance in mg. per 100 c.c. oil.												
	0	- 0·05	—0·1	—0·5	- 1	2	3	4	--5	- 6	- 9	—10	10—
+++	0	1	0	0	0	0	0	4	1	3	1	1	1
++	0	0	1	0	0	0	2	2	0	1	0	0	0
+	0	0	0	0	0	0	2	0	0	1	1	0	0
—	8	11	2	1	0	1	0	0	0	0	0	0	0

It will be observed that with two notable exceptions, viz., samples No. 301 and No. 69 (*see* note below for full details and discussion), there is, on the whole, a remarkable correspondence between the toxicity of the oil as roughly estimated from epidemiological history and its reacting substance content. This by itself is an important finding. It argues for confidence in the quantitative test as a measure of toxicity and emphasizes the fact that the presence of the reacting substance in small amounts is not associated with manifest disease. Leaving aside the two discordant results, for the moment, it would appear that roughly speaking clinical symptoms are likely to appear if argemone oil is present in mustard oil in quantities above one per cent. However, the question of the total amount of the oil taken and the period over which its consumption is spread out must necessarily enter into consideration apart from other possible factors connected with the personal habits and individual constitutions about which we have at present no information. Information regarding the former two points in relation to the development of clinical cases is shown in Tables XVIII-*a* and XVIII-*b*.

Note.—Sample No. 301. Extinction coefficient value 0·018. Toxicity +++.

A Sanitary Inspector collected the sample from the family at a time when the persons were actually ill. All the 10 members of the family except one baby 2 months old and a girl 14 years old were affected. The victims included 5 children under 10 years and one son 11 years old. The children were the first to be affected; the chronological order of cases was roughly in ascending order of age. The illness was serious, the faces of children being involved in œdema. The 11-year-old son got œdema of the lungs and breathlessness. Thus, there can be no question about the high toxicity of the oil if that was the cause of the disease. From the report accompanying the sample it would appear that the oil was purchased in bulk equal to nearly 2 months' consumption and had been consumed at the rate of about 1·2 ounces per person per day for nearly 35 days before the first case occurred. These details would suggest there might have been a cumulative action resulting in severe disease. No information is available as to how the oil was stored before the sample was taken, though the suggestion is that it was probably kept in a tin. Under the circumstances apart from the possibility of wrong labelling the discrepancy in the toxicity as suggested by the epidemiological history and the low value of extinction coefficient cannot be explained.

Sample No. 69. Extinction coefficient value 0·04. Toxicity ++.

The oil of which the sample is assumed to be a part was definitely toxic because 5 out of the 12 members in the family were victims. The head of the family—a practising physician—was among the sufferers. The sample which is properly labelled was collected by a Sanitary Inspector and was transmitted along with other samples by the District Health Officer.

According to the report accompanying the sample only 6 days' supply was left when the first case occurred. Unless the use of this particular supply was stopped at that time, and of this there is no mention, all the oil should have been consumed much before the date of the Sanitary Inspector's visit. It is therefore questionable whether the sample is really a part of the supply of oil which caused the disease.

Table XVIII-*a*.

Number of total and of affected persons (in brackets) in families in relation to the amount of the reacting substance present in the average amount of oil consumed per person and the period of consumption.

Period of consumption of oil in days.	Average amount of the reacting substance in milligrams present in toxic oil consumed during the whole period per person												
	0	—0·1	—0·5	—1·0	—2·0	—5·0	—10·0	—20·0	—30·0	—40·0	—50·0	—60·0	60·0—130·0
82	..	..	..	..	..	..	..	..	..	4 (2)	..	..	..
65	..	..	..	..	..	..	..	..	..	..	..	..	5 (2)
40	..	..	..	..	..	..	..	..	..	..	10 (6)	..	..
35	..	..	9 (8)	..	..	..	..	..	..	..	..	..	..
30	..	..	2 (0)	..	..	..	..	..	..	..	..	..	..
30	..	..	27 (0)	..	..	..	..	..	..	..	..	..	..
30	8 (0)	..	..	..	..	..	..	..	..	..	..	..	..
25	..	15 (0)	..	..	..	..	..	..	..	..	..	..	..
24	..	..	..	..	..	..	..	..	11 (6)	..	..	..	..
21	..	..	..	..	..	..	..	12 (2)	..	..	..	..	..
21	..	..	3 (0)	..	..	..	..	..	..	..	..	..	..
20	..	12 (0)	..	..	..	..	..	..	..	..	..	..	..
20	13 (0)	..	..	..	..	..	..	..	..	..	..	..	..
20	..	..	..	..	..	..	..	24 (7)	..	..	..	..	..
16	..	..	..	..	..	..	..	6 (6)	..	..	..	..	..
16	..	..	..	..	..	..	..	15 (9)	..	..	..	..	..
16	..	..	12 (5)	..	..	..	..	..	..	..	..	..	..
15	..	..	17 (0)	..	..	..	..	..	..	..	..	..	..
15	..	..	3 (0)	..	..	..	..	..	..	..	..	..	..
15	..	..	15 (0)	..	..	..	..	..	..	..	..	..	..
15	9 (0)	..	..	..	..	..	..	..	..	..	..	..	..
15	4 (0)	..	..	..	..	..	..	..	..	..	..	..	..
12	19 (0)	..	..	..	..	..	..	..	..	..	..	..	..
11	..	..	..	..	..	..	..	6 (3)	..	..	..	..	..
9	..	..	..	..	..	..	..	8 (5)	..	..	..	..	..
8	..	..	..	..	..	..	..	7 (4)	..	..	..	..	..
8	..	..	..	..	..	..	11 (6)	..	..	..	..	..	..
8	..	..	..	..	..	..	..	7 (2)	..	..	..	..	..
7	..	..	..	..	14 (7)	..	..	..	..	..	..	..	..
7	..	..	..	..	..	..	..	28 (4)	..	..	..	..	..
6	..	..	..	..	19 (9)	..	..	..	..	..	..	..	..

TABLE XVIII-*b*.

Amount of the reacting substance present in the total amount of oil consumed and the total period of consumption for each experimental subject. Figures (in brackets) represent cases.

Sample number of oil.	Period of consumption of oil in days.	Amount of the reacting substance in milligrams present in toxic oil consumed during the whole period per person.										
		0	—0·2	—0·5	—2·0	—5·0	—10·0	—20·0	—30·0	—40·0	—50·0	—60·0
3	20	..	..	..	..	..	..	..	1 (0)	..	..	..
	20	..	..	..	..	..	..	..	1 (0)	..	..	..
	20	..	..	..	..	..	..	..	1 (0)	..	..	.
13	20	..	..	..	..	1 (0)	..	..	..	..	..	..
	20	..	..	..	..	1 (0)	..	..	..	..	..	..
	20	..	..	..	..	1 (0)	..	..	..	..	..	..
14	19	..	..	1 (0)	..	..	..	..	..	..	..	..
	19	..	..	1 (0)	..	..	..	..	..	..	..	..
	19	..	..	1 (0)	..	..	..	..	..	..	..	..
19	19	..	..	..	..	..	..	1 (0)	..	..	..	..
	20	..	..	..	..	..	..	1 (0)	..	..	..	..
	20	..	..	..	..	..	..	..	1 (1)	..	..	..
20	18	..	..	..	..	..	..	..	..	..	1 (1)	..
	18	..	..	..	..	..	..	..	..	1 (1?)	..	..
	21	..	..	..	..	..	..	..	..	..	..	1 (1)

The results of the experimental subjects (Table XVIII-*b*) are interesting. The period during which the toxic oil was consumed is about the same in all cases (18 to 21 days). Of the four subjects taking oil representing a total of 22 mg. to 23 mg. of the reacting substance each, only one got mild symptoms of the disease while all the eight subjects taking less than this amount escaped, and all the four taking more than that quantity developed symptoms (including one doubtful case). The number of experimental subjects is unfortunately small but the results indicate that consumption of oil representing 1 mg. of the reacting substance per day for 20 days may be attended with certain amount of risk and this quantity perhaps represents about the lowest limit in the case of healthy persons living under hygienic conditions on well-balanced and adequate diets.

The results obtained from the field studies are not so clear cut. In general they suggest that the disease might be caused by oil representing lesser quantity of the reacting substance. Thus, taking a 20 to 21 days period of consumption for purposes of comparison with the experimental subjects smaller quantities of the reacting substance, i.e., less than 20 mg. actually 18·5 in the case of one and 11·6 in the case of the other family, resulted in the development of cases, larger number in the former family than in the latter. Possibly this discrepancy may be explicable on the ground that the persons who actually fell sick had taken more than their due share of the oil or that the reported amount of oil actually consumed had been an understatement or again the families had been taking toxic oil prior to the supply from which the sample was taken. Another possible reason may be that the diet, general health and sanitary conditions were inferior in the field cases, but this explanation is not likely to be correct. Further the data given in Table XVIII-*a* suggest that oil representing smaller quantities of the reacting substance may produce disease symptoms if the concentration of argemone oil is high, thus indicating that the period over which a given quantity of toxic oil is consumed may be a significant factor.

2. *Behaviour of samples of known extinction coefficient value.*

A priori this method should be capable of giving more valuable information because the extinction coefficient value of the oil is determined before it is consumed and the question of the deterioration of the reacting substance does not arise. However, it is not an easy matter to obtain precise information regarding the effects of consumption of the oil. Most of the oil mills and dealers in mustard oil have become aware of the fact that the oil is in some way concerned in the ætiology of epidemic dropsy and it is difficult to obtain their co-operation because they are afraid of prosecution and of loss of business. Moreover, the retail merchant usually mixes different brands of oil before he dispenses it to the consumers. The average householder purchases oils in small quantities only and the source of his oil frequently changes. The clientele of a mustard oil shop being indefinite it is difficult to exclude the existence of the disease amongst them previous to the consumption of oil from a particular consignment or the use of toxic oil from another source which might have contributed towards the development of the symptoms. In view of these difficulties investigations were conducted mainly in two fields :—

(*a*) Students' hostels in which mustard oil is purchased in bulk.

(*b*) The area of distribution of oil from a particular oil mill.

(*a*) *Investigation in students' hostels.*—Through the courtesy of the Calcutta University authorities and members of certain private hostels co-operation of a number of private hostels was secured but it was found that in only eight of them mustard oil was purchased in bulk. The number of inmates varied from 7 to 38, the total population being 120. Data regarding the history of oil in use and the physical examination of the students were entered in a schedule (reproduced in the *Appendix*). A sample of mustard oil in use at the time was collected and if found positive it was submitted to the quantitative chemical test. Visits to the hostel

were repeated every week and the above information and other relevant facts were collected as also a sample of oil if a new supply had been obtained.

These investigations have been carried out over a period of 11 months but neither samples of oil of high extinction coefficient value have been discovered nor any cases of the disease have been seen (*vide* Tables XIX and XX).

TABLE XIX.

Analysis of results of chemical tests on samples of oil collected from students' hostels.

Nitric acid test.	Cupric acetate test. +	Cupric acetate test. −
+	12	19
−	−	123

TABLE XX.

Mustard oil containing small quantities of argemone oil consumed without causing symptom of the disease.

Serial number of samples.	Collected from college hostel or private hostels.	Percentage of argemone oil present.	Total number of persons.	Average quantity of mustard oil consumed per head per day in ounces.	Period of consumption in days.	Remarks.
454	Postgraduate Science hostel, Amherst Street.	0·07	22	1·5	17	No complaints.
423	Private hostel (B), Baitakkhana Road.	0·07	7	1·1	10	„
450	Private hostel, Wellesley Street.	0·08	18	0·9	10	„
404	Private hostel (A), Baitakkhana Road.	0·10	9	1·3	7	„
417	Private hostel (B), Baitakkhana Road.	0·10	7	1·1	10	„
409	Do.	0·15	7	1·1	10	„
422	Private hostel, Suhrawardy Avenue.	0·56	7	1·1	10	„

The results are only negative in character but they are of considerable value inasmuch as they indicate that an ounce of mustard oil containing as much as 0·56 per cent argemone oil may be consumed by an adult over a period of 10 days, while taking the usual Bengali diet, including rice, without the development of manifest signs of the disease. This observation supports the conclusion already arrived at from the study of the field data. As to whether subclinical doses of the toxic material taken over a long period produce some undetectable pathological lesions or predispose the individuals to smaller doses of the toxic substance or to other diseases is quite another matter.

(*b*) *Tracing the consumption of mustard oil from an oil mill.*—This investigation was undertaken on a broader basis. The object here was to trace the process of the development of an actual epidemic of epidemic dropsy from the beginning to the end. This was made possible by the co-operation offered by the management of an oil mill that had sought our assistance in producing good mustard oil so that they might not be subjected to further punishment and might re-establish their reputation and public confidence which had been badly shaken recently. It was arranged that samples of mustard seed which were proposed to be crushed would be daily submitted for examination and pressing will not be proceeded with unless permission to do so had been obtained. A sample of the oil pressed out from each consignment of the seed was also to be submitted for examination. Certain pressing machines, divided into two groups, were specially cleaned and set apart for pressing oil from such supplies of seed as were selected by us. The oil in each of these cases was to be stored in specially cleaned tanks designated 'A' and 'B' and corresponding marks were to be embossed on the tins in which oil from each tank was to be distributed. It was also arranged that these two supplies of oil were to be consigned, as far as possible, to consumers who were in the habit of purchasing oil in bulk and in any case before the tins were despatched, the address of the proposed consignee would be communicated to us and the notice will be given sufficiently early to permit a thorough health survey of the prospective consumers to be made so as to exclude the existence of epidemic dropsy amongst them. But for this last-named condition the authorities of the oil mill strictly followed the above arrangements.

As stated in the first section of this report 328 samples of mustard seed were examined to determine the percentage of argemone seeds present in each sample (*vide* Table I-*a*). A sample of *rai* variety showing the highest percentage of the weed (8 per cent) was selected. The extinction coefficient at 460 M. U. of the oil pressed out of this supply was 2·08 which is equivalent to 2·0 per cent of argemone oil. Before putting in the tins (about 36 lb. capacity) it was mixed with oil pressed from other type of seed; the extinction coefficient value at 460 M. U. of the final mixture was found to be 1·64 (corresponding to 1·55 per cent of argemone oil). These tins numbered 97. They were marked 'A'. Similarly, oil pressed from another supply of mustard seed in which no argemone seed were found was put in tins marked 'B'. Altogether 190 tins, 97 marked 'A' and 93 marked 'B', were produced. As has been stated before the management of the oil mill failed us at this stage and sent out these tins to the customers without giving any information to us. However, information about one consignment of 64 tins sent to Satkhira, a small town in

Khulna district, was received about a fortnight after the date of despatch. The place was immediately visited and an investigation into the articles of trade of all grocers' shops was made. According to the information supplied by the mill the consignment was made to one customer only, namely, a shop in Satkhira which was a regular customer of the manufacturers and which for the sake of convenience may be designated shop 'C'.

On inquiry it was found that a consignment of 64 tins was actually received by shop 'C' on the expected date but at the time of investigation only 17 tins bearing manufacturer's trade mark were found in stock and of these only 12 bore mark 'A'. The shop records showed that 9 tins out of the consignment were sold to two shops one (to be called 'F') in Satkhira (5 tins) and the other (to be called 'E') in Basdah (4 tins). In addition 16 tins had been supplied to other customers whose addresses had not been noted as they had made cash payments. Of these 5 or 7 tins had been purchased by local shops. The remaining 22 tins must have been sold in retail. Other grocers in Satkhira unlike 'C' stocked more than one brand of oil and frequently mixed them before retail sale. While many tins of the particular brand were found in these shops only 1 tin bearing mark 'A' was seen in shop 'F' and 3 in shop 'E', the latter shop having in addition a tin of the same brand but without mark 'A'. In two other shops in Basdah one 'A' marked tin was found in each case and one of these shops stocked oil of other brands at the same time. It would thus appear that the consignment of 64 tins did not entirely consist of tins 'A' though majority of tins belonged to that lot and local inquiry suggested that in shop 'C' and more so in other shops oil marked 'A' underwent admixture with other brands of oil before being actually exposed for sale. Such admixture would further reduce the already low argemone oil content of the oil and tend to bring it down to subclinical level of toxicity as deduced above. Even at the time of investigation when the stock of 'C' consisted mostly of 'A' marked tins there was actual dilution as shown by the quantitative examination of oil samples taken from the tin exposed for sale (*vide* Table XXI).

TABLE XXI.

Extinction coefficient values of samples of mustard oil collected from the shop 'C' at Satkhira.

Sample number.	Date of collection.	Extinction coefficient at 460 M. U.
340, from sealed tins marked 'A' ..	21-1-39	1·70
341, open and exposed for sale ..	21-1-39	1·68
349 ,, ,, ..	26-1-39	1·36

TABLE XXI—*concld.*

Sample number.	Date of collection.	Extinction coefficient at 460 M. U.
350, open and exposed for sale ..	27–1–39	1·36
351 „ „ ..	31–1–39	0·70
352 „ „ ..	3–2–39	0·30

Satkhira is a small subdivisional town with a population of 5,726 distributed in 923 families. A rapid survey of the population, which included 540 children under 3 years of age, showed that there had been a pretty severe epidemic during the last rainy season (86 cases in 70 families of which 3 still showed residual symptoms), besides 27 other cases had occurred within the year and 53 cases beyond one year but within 5 years. No recent cases could be traced at the time of investigation which was carried out within a fortnight of the arrival of the consignment of lot ' A ' oil nor within a month afterwards or to be more precise within 13 days after the last remains of the toxic oil had been sold. No cases were discovered on re-visiting the town on the 5th March.

In order that more definite information following the use of the lot ' A ' oil may be obtained a local literate man was appointed whose duties were to spend the day at shop ' C ' and note the names of the persons purchasing oil. This arrangement lasted for 11 days and was discontinued when lot ' A ' oil had been finished. Table XXII sets out the frequencies of purchases of oil from shop ' C ' by various customers :—

TABLE XXII.

Frequency of purchase of oil from shop ' C ' by various customers in 11 *days.*

Frequency of purchases ..	1	2	3	4
Number of customers ..	3	2	3	4

Table XXII serves to draw attention to the fact that as a rule only a few days' supplies were purchased at a time.

As a result of further investigations carried out in these 12 families 3 had to be excluded from further considerations as they were not regular customers. In another case the head of the family did not acknowledge having purchased oil from shop ' C '. One of the customers obtained oil from two shops at the same time

and, therefore, the quantity of oil purchased from shop 'C' could not be ascertained, in yet another case the purchaser was a hotel keeper whose clientele was not fixed. The details of remaining 6 families are set out in Table XXIII :—

TABLE XXIII.

Probable amount of toxic oil consumed by 6 families purchasing oil from shop 'C'.

Family number.	Period of consumption of oil in days.	Total amount consumed by the family in ounces.	Total amount consumed per head in ounces.	Amount consumed per head per day in ounce.
287	28	100	20	0·71
291	28	320	27	0·97
97	21	105	15	0·71
128	28	288	32	1·14
198	30	320	25	0·83
69	30	320	25	0·83

In making the calculations children under 3 years have been excluded and attempt has been made to include the period during which toxic oil only was presumably consumed. No symptoms of the disease developed amongst the members of these families during the period of observation which extended over 5 weeks after the supply of oil had been exhausted in shop 'C'. We may provisionally conclude that on an average consumption of 8 ounces of mustard oil per head containing 1·55 per cent (?) of argemone oil may be consumed by a family (in this case a family of 9 persons over 3 years) living on typical Bengali diet for a period of 28 days without causing demonstrable symptoms of the disease. A further point of interest is that 3 out of these 6 families had been involved in the outbreak which had occurred 6 months previously. This fact would indicate that the oil of such toxicity (?) as lot 'A' would not cause even a recurrence of the disease amongst those who had recently suffered from it if taken in such small doses.

In Basdah where about 300 families of poor weavers and cultivators reside shop 'E' sells on an average about 1½ tins of oil every month, most of the sales being affected twice a week on market days. It could count only 4 families as its regular customers, the amount of consumption of oil being only about 1 to 2 ounces a week per head. None of the members of these families suffered from the disease at the time of investigation nor within a month afterwards.

These observations would have argued against the oil being capable of causing symptoms of the disease had it not been for the fact that the disease attributable to this lot of oil actually broke out amongst members of a well-to-do family which

had been personally supplied with a full tin by the manager of the mill who had of course not been told about the danger associated with its consumption. Thus, although we were disappointed in not being able to trace the whole history of an epidemic beginning with the discovery of argemone seed in the stock of mustard seed to the development of an epidemic attributable to the oil expressed from that stock this investigation served for the purposes of a detailed study of the actual conditions of distribution of oil in a small town and a village and of supplying rough quantitative data about the relationship between the toxic substance in the oil with disease manifestation under natural conditions.

3. *The experimental studies.*

As previously mentioned these studies should have yielded the most valuable information on the problem under consideration but for the fact that the quantitative test was developed long after some of the best experimental studies had been made and consequently the samples of oil employed had been exhausted or had become very old. However the available data are set out in Table XXIV :—

TABLE XXIV.

Quantitative estimates of argemone oil in samples of mustard oil used in the human experiments.

Sample number.	Percentage of argemone oil present.	Total number of persons.	Average quantity consumed per head per day in ounces.	Average period of consumption in days.	Clinical result.	Time interval, in months, between the commencement of the feeding experiments and the date of the quantitative examination of the oil.	REMARKS.
14	0·01	3	2·04	19	No case	11	*Vide* Part VIII of this series (*I. J. M. R.*, July 1939).
13	0·12	3	2·25	20	No case	14	do.
3	0·80	3	2·23	20	No case	14	Part VII of this series (*I. J. M. R.*, July 1939).
19	1·27	3	1·55	17	One case	13	do.
20	4·00	3	1·19	17	Two cases and one doubtful.	13	do.

Here again the results correspond with those obtained in previous sections, viz., that 0·8 per cent argemone oil in mustard oil taken at the rate of 2·33 ounces

daily over a period of 20 days did not give rise to symptoms of the disease in adult males, but the use of 1·55 ounces of mustard oil containing 1·27 per cent of argemone oil taken for 17 days was just sufficient to cause very mild symptoms.

Discussion.

These observations suggest that the families mentioned in connection with the Satkhira investigations were just lucky to escape as according to the information contained in the table they had probably consumed toxic substance in amounts just short of causing symptoms. Although the lot 'A' oil contained slightly higher percentage of argemone oil than sample 19 in Table XXIV, the quantities consumed per head per day were smaller and it had probably undergone dilution with non-toxic oil during greater part of the period of consumption by the families (*vide* Table XXI). Another interesting point which emerges from these studies is that well-balanced and adequate food does not afford any protection against the disease and the recent speculations on the subject (Editorial, *Ind. Med. Gaz.*, October 1937) are not well founded.

It would be fair to state that in spite of the highly involved nature of the problem it may be safely concluded that the presence of less than 1 per cent of argemone oil in mustard oil will fail to cause demonstrable symptoms of epidemic dropsy under reasonably average conditions of mustard oil consumption in Bengal and after allowing for the possible subclinical effects of the toxic substance taken in smaller doses it would be reasonable to say that the use of mustard oil containing less than 0·5 per cent of argemone oil as demonstrated by the quantitative test described here may be permitted without danger of developing epidemic dropsy. This standard will probably cover abnormal cases in which large quantities of oil are consumed or the oil of low toxicity is taken over prolonged periods. In accepting this standard it should be insisted that the oil submitted for examination is received as soon after it is exposed for sale as possible and that it should be transmitted to the analyst in dark glass phials which should be as completely filled as possible to exclude air. In laying down the standard it has been assumed that small quantities of the toxic substance do not do any harm. This assumption may or may not be strictly correct and possibly at a future date it might be shown that continued use of toxic substance over prolonged periods causes some harmful effects, such as lowering resistance of mucosa or skin against infection. However, at present these are not more than mere speculations and until some definite evidence against the use of oil containing small quantities of the toxic substance has been adduced it may be safely recommended that a standard of less than 0·5 per cent of argemone oil in mustard oil may be adopted for legal purposes. With reasonable care taken by the managements of oil mills in selecting mustard seed, few if any samples of oil need be rejected.

Summary.

1. It has been shown that many stocks of mustard seed, particularly those of *B. juncea* found in oil mills, contain variable amounts of argemone seed. In some of these supplies the proportion of argemone seed, though sufficient to give rise to

positive results with nitric acid test in the oil expressed from them, may not be enough to cause symptoms of the disease. It is therefore a question whether a wholesale rejection of such supplies would be justifiable and whether it would not unnecessarily dislocate business. Quantitative aspect of the problem, therefore, assumes a considerable importance.

2. A colorimetric quantitative test has been developed and gauging curves have been worked out from which milligrams of the reacting substance per 100 c.c. of oil and roughly the percentage of argemone oil can be directly read off against the colorimetric (Pulfrich) reading.

3. It has been shown that light and air reduce the reacting-substance content of mustard oil containing argemone oil.

4. While light is necessary for this reaction, presence of air is not essential in the case of mixtures of argemone oil in mustard oil. It is, however, necessary in the case of pure argemone oil.

5. Both the visible and ultra-violet radiations act equally well in reducing the reacting-substance content of the oil. Direct light gives quicker results than diffused light.

6. Heat is not concerned in this reaction.

7. Attempt has been made to determine the minimum quantity of argemone oil in mustard oil the consumption of which will produce symptoms. The problem has been tackled in various ways, namely, comparison of the toxic effects, as suggested by the epidemiological histories, with the reacting-substance contents of the oil, tracing an oil containing known amount of reacting substance to the consumers and observing developments amongst them, concurrent study of the oil and of the persons consuming it and finally determining the reacting substance of samples of oil used in human feeding experiments.

All these studies point to the conclusion that oil containing less than one per cent argemone oil, or oil representing 1 mg. of the reacting substance taken daily for 20 days, is not likely to produce clinical symptoms.

Conclusions.

1. The number of stocks of mustard seed in oil mills containing small amount of argemone seed is so large that their indiscriminate rejection will cause considerable dislocation of the industry.

2. A quantitative chemical test has been developed and gauging curves have been presented by means of which the reacting-substance content of oil and roughly the percentage of argemone oil can be determined in samples of mustard oil.

3. It has been shown that the reacting-substance content of toxic mustard oil diminishes under the action of light (diffused and direct) particularly in the presence of air. Air alone does not cause this reduction.

4. It is recommended that sale of mustard oil containing 0·5 per cent or more of argemone oil, for human consumption, should not be permitted. For purposes of the quantitative test it is necessary to submit fresh samples of oil in dark-coloured phials which should be filled to the brim to exclude air.

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APPENDIX.

Form for diet survey used in certain Calcutta hostels.

Date :

Serial number.	Number and identification.	Date of collection of oil.	Affected.	Unaffected.	Date of purchase by hostel.	Quantity purchased of which sample is a part.	Monthly consumption.	Date of 1st case.	Total persons.	Total cases.

Serial number.	Name.	Date of joining.	MEALS. In.	MEALS. Out.	History of E. D.		Pulse.	Heart sounds.		Pigmentation.		Diarrhœa.		Eyes.	Œdema of feet.		Case number.	Remarks.
			One or both.		Yes.	No.		Normal.	Abnormal.	Yes.	No.	Yes.	No.		Yes.	No.		
1																		
2																		
3																		
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HÆMATOLOGICAL CHANGES IN EPIDEMIC DROPSY.

BY

P. C. SEN GUPTA, M.B.,
Assistant Research Worker,

AND

L. EVERARD NAPIER, F.R.C.P. (Lond.),
Professor of Tropical Medicine.

(From the School of Tropical Medicine, Calcutta.)

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HÆMATOLOGICAL changes in epidemic dropsy have been studied by Ray (1927) and Chatterjee and Halder (1935) in the recent years, but the conclusions arrived at by these workers differ from each other in important details. The present study was undertaken to determine the nature of the blood changes in epidemic dropsy by modern standard methods of hæmatology. The data collected have been analysed and compared with known standards for Indian population, in order to determine their significance.

The investigations were carried out amongst the Indian male and female patients who attended the general out-patients' department of the Calcutta School of Tropical Medicine. The patients were residents of *bustees* in the north-eastern suburbs of Calcutta, and almost all of them belonged to the labourer class. Every one of these patients presented the typical syndrome of epidemic dropsy; the duration of illness varied from 6 days to 2 months. The hæmatological investigations carried out consisted of : (*a*) study of the nature of anæmia associated with epidemic dropsy, (*b*) the determination of the sedimentation rate and (*c*) study of the changes in the leucocyte counts, total and differential. All the examinations, except the differential counts, were made from oxalated* venous blood, collected in the forenoon.

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* Five c.c. of blood is obtained from a vein and is placed in a small bottle containing 4 mg. solid potassium oxalate and 6 mg. ammonium oxalate in the dry state; with this mixture no correction for shrinkage is necessary.

INVESTIGATION OF ANÆMIA ASSOCIATED WITH EPIDEMIC DROPSY.

This included estimation of hæmoglobin content (by Hellige normal hæmometer), enumeration of red cells per cubic millimetre of blood, reticulocytes per cent of red cells, determination of total cell volume and calculation of mean corpuscular volume (MCV), mean corpuscular hæmoglobin (MCH), and mean corpuscular hæmoglobin concentration (MCHC) of the red cell, and the van den Bergh test. Blood smears were examined for the presence of abnormal red cells. The results of blood counts in 11 male and 11 female patients are shown in *Protocols I* and *II* (*see* end of paper). Analysis of the figures shows the following mean values and standard deviations (*see* Table I).

TABLE I.

Analysis of hæmatological findings.

	MEAN VALUES WITH STANDARD DEVIATION.	
	Males.	Females.
Hæmoglobin in g. per 100 c.c. ..	9·43 ± 2·13	8·29 ± 1·91
R.b.c. in millions per c.mm. .	3·32 ± 0·75	2·95 ± 0·86
Reticulocytes per cent of red cells ..	0·509 ± 0·478	0·47 ± 0·30
MCV (cu.μ)	85·3 ± 6·7	88·41 ± 10·21
MCH (γγ)	28·49 ± 2·4	28·57 ± 2·02
MCHC (per cent)	32·3 ± 1·26	32·06 ± 1·92
Van den Bergh test . ..	Negative, i.e., bilirubin content within normal in all cases.	

The hæmatological values for 'normal' Indian males and females of this particular class is not known definitely. Napier and his co-workers found the hæmatological levels for 'normal' Indian middle-class population, and 'normal' Indian females of Calcutta, and of the Assam coolies, male and female (*see* Table II). It is probable that the values for the 'normal' population of Calcutta *bustees* would be intermediate between these two sets of figures, as far as the hæmoglobin percentage is concerned. The poorer-class hospital patient always tends to have a smaller cell than the standards given for the middle-class Calcutta resident, but certainly not usually as small as that of the Assam coolie. A comparison of the figures obtained in this present study with either of the sets of figures shows that

a definite degree of anæmia is produced in epidemic dropsy. This is in accordance with the findings of the previous workers.

TABLE II.

Hæmatological findings in 'normal' Indians.

Mean values and standard deviations.

	Hb. in g. per cent.	R.b.c. in millions per c.mm.	MCV (cu.μ).	MCH (γγ).	MCHC (per cent).	Source.
'Normal' Indian males in Calcutta (middle-class population).	15·7 ± 0·91	5·53 ± 0·49	90·49 ± 7·9	28·53 ± 2·31	31·07 ± 1·20	Napier and Das Gupta (1936).
'Normal' Indian females in Calcutta.	12·58 ± 1·01	4·61 ± 0·41	86·82 ± 7·28	27·42 ± 2·89	31·57 ± 1·76	Napier (1939).
'Normal' Assam coolie population. Males.	12·63 ± 1·42	5·27 ± 0·71	71·29 ± 7·04	23·93 ± 2·31	32·50 ± 3·10	Napier and Das Gupta (1936).
'Normal' Assam coolie population. Females.	11·30 ± 1·19	4·93 ± 0·61	72·30 ± 8·80	23·35 ± 2·40	33·07 ± 2·55	do.

TYPE OF ANÆMIA.

Ray (*loc. cit.*) was of opinion that the anæmia was of the 'secondary' type, the colour index being less than normal. Chatterjee and Halder (*loc. cit.*) stated that the diminution of hæmoglobin was more pronounced than that of the total number of red blood cells, especially in early cases, meaning thereby that the anæmia was hypochromic in nature. The data arrived at in the present study, which included determination of absolute values of the red cell, viz., MCV, MCH, MCHC, and therefore allowed a more accurate determination of type of anæmia, do not confirm the views of the previous workers.

Detailed consideration of the protocols and tables shows that of the males two cases are slightly macrocytic, one slightly microcytic and the remaining eight normocytic in type. Of the females three are slightly macrocytic, two microcytic and the rest normocytic. In only one male and two females were the cells hypochromic. The anæmia in the average case has been found to be orthochromic and normocytic or slightly macrocytic in type in both sexes. In view of the fact that the poorer-class hospital patient has a smaller cell than the average of the

middle-class Calcutta population, the average size of the red cell in the epidemic dropsy group is probably just above the 'normal' of the particular class.

Mechanism of production of anæmia in epidemic dropsy.

The van den Bergh test (indirect) done in this series of cases was negative in every instance, i.e., the bilirubin content was never found to be above the normal limits, nor was there any reticulocytosis. This suggests that there is little excess blood destruction in this condition. Also in no case of the series was there a history of bleeding previous to the blood count. So the anæmia in these cases is apparently not due to excessive blood loss. The other alternative is that the anæmia is due to depression of erythropoiesis.

In this connection it is worth while considering the cytological counts in sternal marrow biopsy of three cases of epidemic dropsy admitted in the Carmichael Hospital for Tropical Diseases (*see* Table III).

TABLE III.

Sternum puncture findings.

	Case I B. G.	Case II D. G.	Case III U. N. S.
Total red cells (millions/c.mm.) ..	1·88	2·15	2·58
Reticulocytes (per cent of r.b.c.) ..	0·9	5·2	2·9
Hæmoglobin (g. per cent)	5·6375	6·6	6 325
Total nucleated cells (thousands/c.mm.)	16·0	37·5	53·0
Red cell series per cent ..	*37·2*	*38·8*	*28·8*
Megaloblast ,, ..	0·4	2·4	0·4
Erythroblast ,, ..	2·0	2·0	0·4
Normoblast ,, ..	34·8	34·4	28·0
White cell series ,, ..	*62·8*	*61·2*	*71·2*
Myeloblast ,, ..	*Nil*	*Nil*	0·4
Premyelocyte ,, ..	0·4	0·8	1·2
Myelocyte: Neutro ,, ..	2·0	3·6	3·6
,, Eosinophil ,, ..	0·8	0·8	2·0
,, Basophil ,, ..	*Nil*	*Nil*	*Nil*
Neutrophil: Young ,, ..	4·8	4·4	18·0
,, Band ,, ..	26·4	20·0	25·6

TABLE III—*concld.*

	Case I B. G.	Case II D. G.	Case III U. N. S.
Neutrophil : Segment per cent ..	14·8	10·8	11·2
Eosinophil „ ..	3·6	6·8	2·4
Basophil „ ..	*Nil*	*Nil*	*Nil*
Lymphocyte „ ..	6·4	7·6	3·2
Large mononuclear „ ..	2·4	6·0	2·0
Plasma cell „ ..	1·2	0·4	1·6
Others „ ..	*Nil*	*Nil*	*Nil*

It will be seen that, though all the cases are markedly anæmic, the marrow is not hyperplastic and the proportion of cells of red cell series is only a little above the normal. The total nucleated-cell count is below normal in one case and within normal limits in the other two. The proportion of cells of red cell series is usually much higher in anæmias where the marrow retains the normal degree of activity. In a majority of cases of anæmias showing normoblastic hyperplasia the total nucleated cell count is over 50,000 and the normoblasts 30 to 50 per cent and over (Napier, *loc. cit.*). These findings together with a low reticulocyte count seen in peripheral blood and the fact that there is no evidence of increased blood destruction or loss, tend to suggest that the anæmia of epidemic dropsy is due to depression of blood formation.

SEDIMENTATION RATE IN EPIDEMIC DROPSY.

The sedimentation rate (SR) was determined according to the Westergren technique. Readings were taken at the end of one hour. The rate was determined in 34 cases (*see* Table IV). It was found that the rate was above normal in all the cases. The sedimentation rate is increased in anæmia, the increase being correlated with the cell volume, so that it is necessary to make an allowance for this fact. Hynes (quoted by Whitby and Britton, 1937) has worked out a chart from which it is possible to ascertain the significance of any sedimentation reading if the cell volume is known.

The cell volume was determined in 21 cases, 11 males and 10 females. It was found that of the 11 male patients six had increased sedimentation rate, in one case it was doubtful and in four the rate was normal when corrected for anæmia. Amongst the females, seven cases had increased rate and three normal rate when corrected for anæmia. It is thus seen that in about 70 per cent of cases in which the correction for anæmia was made there is an increase of sedimentation rate beyond what is caused by anæmia.

The following conclusions regarding the sedimentation rate in epidemic dropsy seem justified :—

(*a*) The SR is increased in all cases of epidemic dropsy and very high figures may be obtained.

(*b*) The increase in the SR is due partly to anæmia that is associated with epidemic dropsy.

(*c*) There are some other factors (in addition to anæmia), toxic or otherwise, that causes this increase in the SR in epidemic dropsy.

Pasricha, Lal, Malik and Biswas (1939) have also noted the rise in the SR in epidemic dropsy.

TABLE IV.

Sedimentation rate in epidemic dropsy.

Serial number.	Sex.	SR in one hour.	Cell volume per cent of blood.	Significance.	Serial number.	Sex.	SR in one hour.	Cell volume per cent of blood.	Significance.
1	M	62	28·0	+++	18	F	78	28·5	+++
2	M	25	30·0	N	19	M	53	..	..
3	F	75	..	..	20	F	30	18·2	N
4	M	41	38·0	++	21	F	64	..	..
5	M	45	37·5	+++	22	F	62	..	..
6	M	54	21·5	±	23	M	116	..	..
7	F	26	27·0	N	24	M	74	32·5	+++
8	M	16	29·0	N	25	F	70	22·5	+++
9	F	55	..	..	26	M	45	..	..
10	M	27	19·0	N	27	M	83	34·2	+++
11	F	146	..	..	28	M	23	23·0	N
12	F	32	38·2	+	29	M	56	..	..
13	F	65	28·75	+++	30	F	15	27·4	N
14	F	74	18·75	+++	31	F	70	26·0	..
15	F	62	..	..	32	F	70	..	..
16	F	12	..	..	33	F	65	22·0	++
17	M	64	..	..	34	M	98	26·75	+++

N = Normal for anæmia.
± = Doubtful.
+ = Slightly increased.
++ = Moderately increased.
+++ = Markedly increased.

Leucocytes in epidemic dropsy.

Ray (*loc. cit.*) was of opinion that there was a distinct leucocytosis in epidemic dropsy. Analysis of his figures shows that the mean total leucocyte count is 8·6 thousands per c.mm. with a standard deviation of ± 4·4 thousands. He regarded about 5 to 6 thousands per c.mm. as normal for Bengalis. Chatterjee and Halder (*loc. cit.*) found that leucocytes were increased early in the disease, later the number remained about normal. *Protocols I* and *II* show the result of the total and differential counts, Arneth-Cooke index and weighted mean of the present series of cases. Analyses of these figures give the following mean values and standard deviations (*see* Table V).

Table V.

Analysis of leucocyte counts.

	Males.			Females.		
	Mean values with standard deviation.			Mean values with standard deviation.		
Leucocytes in thousands per c.mm.	9·05 ± 2·93			7·89 ± 1·34		
Polymorphonuclear (per cent).	62·4 ± 11·43			67·01 ± 9·91		
Lymphocyte (per cent)	24·61 ± 10·67			21·34 ± 8·6		
Large mononuclear (per cent).	5 77 ± 1·73			5·6 ± 1·52		
Eosinophil (per cent)	7·77 ± 2·87			5·67 ± 2·18		
Others (per cent)	Myelocyte	0·4 per cent	in 1 case	Myelocyte	0·4 per cent	in 2 cases.
					0·8 „	in 1 case.
	Basophil	0·4 „	in 5 cases	Basophil	0·4 „	in 2 cases.
		1·6 „	in 1 case		0·8 „	in 1 case.
Arneth index	84·5 ± 8·1			88·36 ± 4·30		
Weighted mean	1·83 ± 0·3			1·81 ± 0·23		

The above tables show that the total leucocyte count is occasionally slightly above the normal, but usually within the normal limits. The differential count in an average case does not show any great deviation from normal limits. The eosinophil count might be considered to be increased on some standards, but Napier and Das Gupta (1935) found a mean of 7 per cent in Calcutta, and values as high as 9 or 10 per cent may be found in apparently 'normal' people. The high Arneth-Cooke index and the low weighted mean show that there is a 'shift

to the left' of the polymorphonuclear leucocytes. This 'shift to the left' in association with a slight degree of leucocytosis or a normal w.b.c. count is indicative of toxæmia.

SUMMARY.

Study of hæmatological changes in epidemic dropsy have shown that epidemic dropsy gives rise to a distinct degree of anæmia, which is usually orthochromic normocytic or slightly macrocytic in type.

The anæmia is caused by depression of erythropoiesis.

The sedimentation rate is increased in epidemic dropsy, the increase is due partly to the associated anæmia and partly to some other factor, toxic or otherwise, in addition to the anæmia.

The mean total leucocyte count is occasionally slightly above normal. The differential count does not show any marked deviation from normal, but there is a distinct 'shift to the left' of the polymorphonuclears.

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PROTOCOL I.

Hæmatological findings in epidemic dropsy.

Males.

Serial number.	Initials.	Duration of illness, weeks.	Hb. in g. per 100 c.c. of blood.	R.b.c. in millions per c.mm.	Reticulocytes per cent of r.b.c.	CV (per cent).	MCV (cu.μ).	MCH (γγ).	MCHC (per cent).	Indirect van den Bergh test.	W.b.c. in thousands per c.mm.	Polymorphonuclear.	Lymphocyte.	Large mononuclear.	Eosinophil.	Others.	Arneth-Cooke index.	Weighted mean.
1	M. A.	2	8·25	3·23	1·0	28·0	86·6	25·5	29·4	Negative	13·0	67·0	24·0	5·0	4·0	Normoblasts + a few.	85·0	1·92
2	S. A.	2	9·48	3·4	0·6	30·0	88·2	27·9	31·29	Negative	9·5	55·0	30·0	6·0	9·0	..	71·5	2·34
3	A. R.	$\frac{6}{7}$	12·65	4·07	0·2	38·0	95·0	31·6	33·2	Negative	10·8	74·4	12·0	6·8	6·4	Basophil 0·4	74·0	2·28
4	J.	$\frac{6}{7}$	12·65	4·83	0·1	37·5	77·6	26·1	33·7	Negative	11·0	55·2	24·0	8·4	12·4	..	88·0	1·8
5	G. M.	4	7·28	2·64	0·3	21·5	81·4	27·6	33·89	Negative	12·0	67·2	15·6	5·2	12·0	..	90·0	1·73
6	N.	$1\frac{5}{7}$	9·21	3·46	0·5	29·0	83·8	26·6	31·7	Negative	8·1	76·0	15·2	4·0	4·4	Basophil 0·4	71·5	2·27
7	R.	1	6·1875	2·14	1·0	19·0	88·7	28·6	32·5	Negative	5·4	36·0	49·6	6·0	8·0	Basophil 0·4	87·0	1·8
8	M. K.	6	10·45	3·83	0·1	32·5	84·8	27·2	32·1	Negative	7·0	62·4	27·6	2·0	6·4	Basophil 1·6	89·5	1·8
9	G. M.	4	11·1375	3·33	0·1	34·2	102·7	33·4	32·5	Negative	12·0	61·2	22·4	7·6	8·4	Basophil 0·4	94·0	1·59
10	J.	6	7·56	2·6	1·6	23·0	88·4	29·08	32·8	Negative	6·1	55·2	33·6	6·0	4·8	Myelocyte 0·4	91·0	1·71
11	J. A.	4	8·9375	3·0	0·1	26·75	89·1	29·8	33·4	Negative	4·7	66·8	16·8	6·4	9·6	Basophil 0·4	87·5	1·87

PROTOCOL II.

Hæmatological findings in epidemic dropsy.

Females.

Serial number.	Initials.	Duration of illness, weeks.	Hb. in g. per 100 c.c. of blood.	R.b.c. in millions per c.mm.	Reticulocytes per cent of r.b.c.	CV (per cent).	MCV (cu.μ).	MCH (γγ).	MCHC (per cent).	Indirect van den Bergh test.	W.b.c. in thousands per c.mm.	Polymorphonuclear.	Lymphocyte.	Large mononuclear.	Eosinophil.	Others.	Arneth-Cooke index.	Weighted mean.
1	R. ..	2½	9·075	2·7	0·4	27·0	100·0	33·6	33·6	Negative	8·9	74·8	13·6	4·8	6·8	..	82·0	2·02
2	P. ..	4	12·5125	4·6	0·3	38·2	83·04	27·2	32·7	Negative	9·0	59·2	26·8	6·4	7·6	..	80·5	2·14
3	U. D. ..	4	9·35	3·0	0·1	28·75	95·8	31·1	32·2	Negative	9·0	60·0	31·6	6·0	2·4	..	89·0	1·78
4	R. K. ..	4	6·46	2·08	0·3	18·75	93·7	32·3	34·9	Negative	7·8	44·8	40·0	7·6	7·2	Myelocyte 0·4	87·5	2·28
5	H. ..	5	8·25	2·82	0·6	..	..	29·2	..	Negative	7·0	66·4	22·8	4·4	6·4	..	89·0	1·73
6	K. B. ..	4	8·525	3·86	0·4	28·5	73·8	22·08	29·9	Negative	7·5	70·4	15·6	8·4	5·2	Basophil 0·4	87·0	1·74
7	S. ..	4	5·225	2·04	0·6	18·2	91·0	26·12	28·1	Negative	6·0	70·8	20·0	5·6	2·8	Myelocyte 0·8	89·0	1·74
8	J. ..	10	7·5625	3·25	1·2	22·5	69·2	23·2	32·6	Negative	10·0	76·0	13·2	5·6	5·2	..	89·0	1·73
9	M. ..	12	8·9375	2·97	0·2	27·4	92·2	30·1	32·6	Negative	6·1	81·6	12·0	2·8	2·8	Basophil 0·8	90·0	1·64
10	J. B. ..	2	8·525	2·66	0·5	26·0	97·7	32·05	32·8	Negative	7·7	68·4	18·8	4·8	7·2	Myelocyte 0·4 Basophil 0·4	95·0	1·52
11	S. B. ..	12	6·875	2·52	0·6	22·0	87·3	27·3	31·2	Negative	8·3	65·6	20·4	5·2	8·8	..	94·0	1·6

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HÆMATOLOGICAL STUDIES IN INDIANS.

Part XII.

HÆMOGLOBIN STANDARDS IN CHILDREN AND ADOLESCENTS.

BY

L. EVERARD NAPIER, F.R.C.P. (Lond.),

AND

C. R. DAS GUPTA, M.B. (Cal.), D.T.M.

(From the School of Tropical Medicine, Calcutta.)

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An opportunity arose to examine a number of Indian boys and girls of different ages. They were all students from different schools in Calcutta and were composed of Bengali Hindus, Indian Christians and Sikhs resident in Calcutta. Their ages varied from 4 to 20 years. The hæmoglobin percentages were estimated by a standardized Hellige apparatus and recorded in grammes of hæmoglobin per 100 c.c. of blood. The weights and heights were taken and these are also analysed in Table I.

First series.—These were girl students from London Mission School, Calcutta. They were Indian Christians and Bengali Hindus, day scholars and boarders, from middle-class families. Their ages varied from 4 to 20 years, but, in the extreme lower and upper age groups, there were so few girls that we have grouped two or more age groups together; the age groups from 6 to 16 are however analysed separately.

Hæmoglobin.—Practically all the means fall between the 11-gramme and the 12-gramme line, but the general tendency is upwards with the age. There is only a small number of individuals in some of the age groups, so that a smooth curve could not be expected. At 9 years the curve almost reaches the 12-gramme line and this level is maintained for three age groups; afterwards it becomes irregular and does not actually pass the 12-gramme line before the oldest

group, 17 to 20 years, is reached. This check in the curve at 11 years is possibly due to the commencement of menstruation, but is a little early for this.

TABLE I.

Age group.	Number in group.	Mean hæmoglobin in grammes.	Mean weight, lb.	Mean height.	
				Feet.	Inches.
4 and 5	8	11·09	34	3	6½
6	14	11·29	37¾	3	7¾
7	10	11·07	40	3	8¾
8	9	11·40	50	3	11¼
9	24	11·84	54	4	2¾
10	18	11·84	60	4	4½
11	15	11·87	71½	4	8¾
12	15	11·37	68¼	4	8¾
13	26	11·98	85	4	11½
14	24	11·39	87½	4	11½
15	22	11·77	89¾	4	11
16	13	11·85	90¼	5	0
17–20	10	12·17	96	5	1

CORRELATION BETWEEN HÆMOGLOBIN, HEIGHT AND WEIGHT.

The hæmoglobin level has a slight upward tendency, that is, it is correlated positively with the age; height and weight are also naturally correlated positively with age, and there is therefore a tendency for hæmoglobin to show some correlation

with both height and weight The point, however, that we wish to ascertain is whether height and/or weight, independently of age, are correlated with the hæmoglobin level. The false correlation can be eliminated to some extent if this point is investigated for each age group separately.

As a rough method of testing the correlationship between height and hæmoglobin level and weight and hæmoglobin level, we first ascertained the mean height of each group, then divided the children into two groups according to whether they were above or below the average height, and took the mean hæmoglobin of each of these two groups. We repeated this procedure, substituting weight for height.

There is no evidence of any constant positive correlation. The height shows a positive correlation in 9 out of 13 age groups, and the weight in 6 out of 13. The early age groups showed a positive correlation more frequently.

TABLE II.

Mean hæmoglobin in grammes per 100 c.c. of blood in persons above and below the mean height and mean weight, respectively, in each age group.

Age in years.	Above mean height.	Below mean height.	Above mean weight.	Below mean weight.	Correlation between hæmoglobin and	
					height.	weight.
4 and 5	11·52	10·66	11·52	10·55	+	+
6	11·29	11·14	11·34	11·00	+	+
7	11·17	10·66	11·52	10·86	+	+
8	11·43	11·00	11·00	11·58	+	–
9	11·74	11·86	12·11	11·74	–	+
10	12·07	11·69	11·48	11·95	+	–
11	12·03	11·69	12·03	11·76	+	+
12	11·19	11·52	11·00	11·48	–	–
13	11·69	12·20	12·20	12·20	–	–
14	11·56	11·48	11·07	11·58	+	–
15	11·88	11·55	11·55	11·81	+	–
16	11·55	12·03	11·28	12·21	–	–
17–20	12·24	12·03	12·38	11·69	+	+

Second series.—The next population to be investigated was at a boys' school in Calcutta, the Tirthapati Institution. The boys, aged from 8 to 18 years, are Hindus of middle-class families and live at home. The early age groups each had

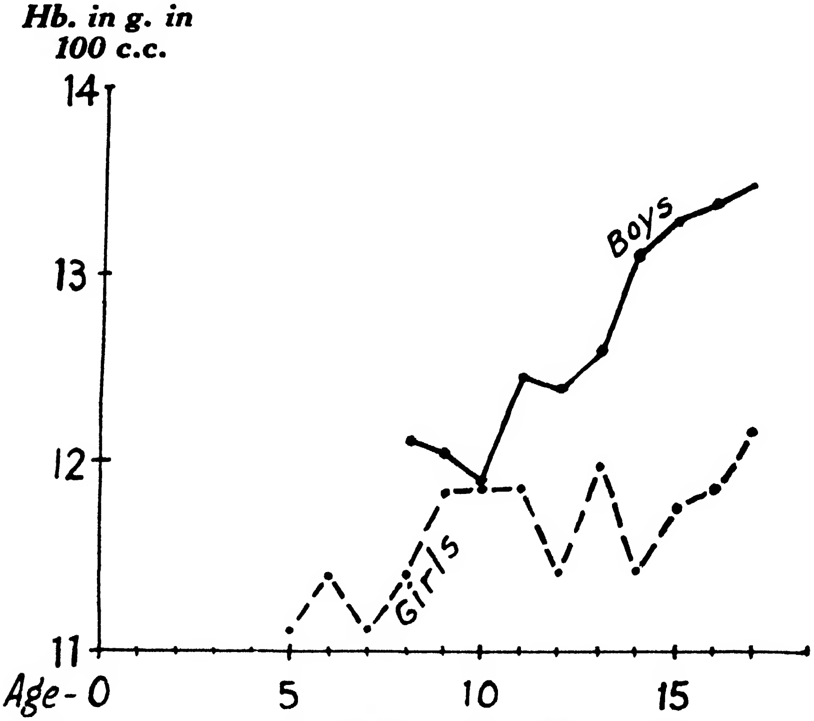

FIG. 1.—Showing the mean hæmoglobin level of Bengali boys and girls of various age groups.

60 or more boys, but the last three age groups are small and have been bunched together. The hæmoglobin curve is shown in Fig. 1. It will be seen that from the age of 10 years the curve rises steadily and sharply.

CORRELATION BETWEEN HÆMOGLOBIN, HEIGHT AND WEIGHT.

On this occasion we reversed the procedure* and to ascertain the correlation we calculated the mean hæmoglobin of each age group. There is a positive correlation between hæmoglobin and both height and weight (Table III and Fig. 2). In the

FIG. 2.—Weight of boys in Tirthapati Institution with hæmoglobin levels above •——• and below —•—•— the average of their own age groups.

higher age group the correlation between hæmoglobin and weight is most marked. By reversing this process (for the weight only), it can be shown that in all the age

* This was due to a misunderstanding on the part of the computator, but the mistake did not seem to be sufficiently important to warrant re-calculation of all the tables.

groups the mean hæmoglobin of those over the average weight of their group is distinctly higher than that of those under average weight. The hæmoglobin curves

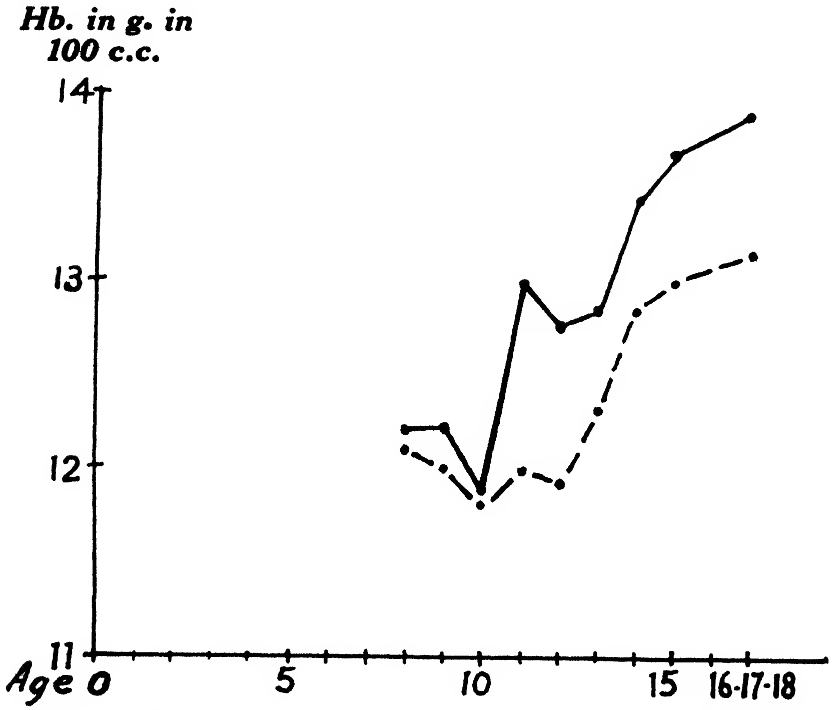

FIG. 3.—Hæmoglobin level at various ages of two groups of boys with weights, respectively, above •——• and below — • — • — the mean of their own age groups.

of the two sets are shown in Fig. 3. In most instances the difference is statistically significant. It is thus apparent that the better-nourished boys have a distinctly higher hæmoglobin.

Third series.—These boys were day scholars at the Khalsa High School, Calcutta. Their parents were all Punjabi Sikhs of the artisan class, carpenters, mechanics and taxi drivers. The age-hæmoglobin curve of the boys is shown in

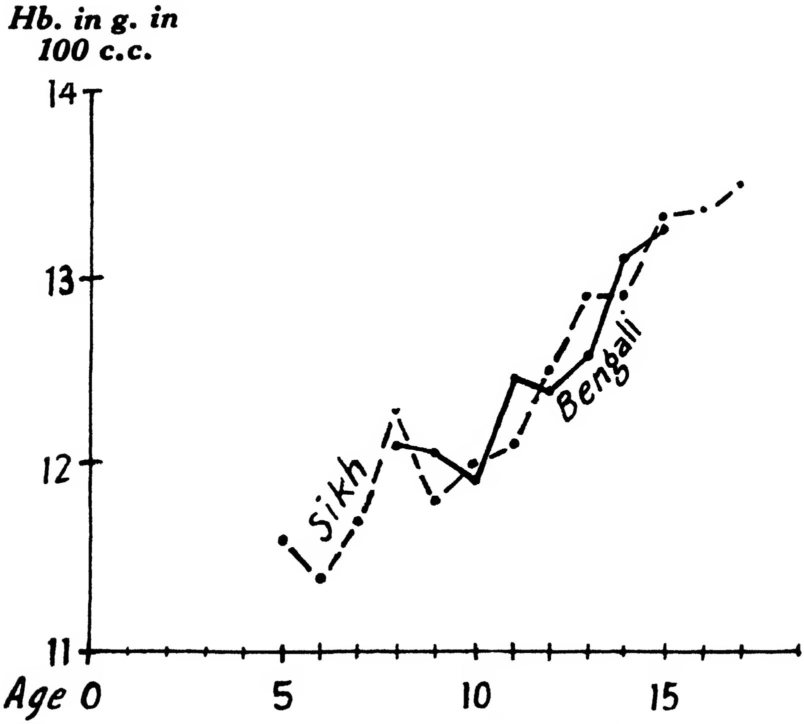

FIG. 4.—Showing the hæmoglobin level at various ages of the two groups of boys, Sikhs and Bengalis.

Fig. 4, and the correlations of hæmoglobin, height and weight in each age group in Table IV and of the hæmoglobin and weight in Fig. 5.

In contrast to the boys of the second series, there is no evidence of any constant correlation between hæmoglobin and either height or weight. This is well shown

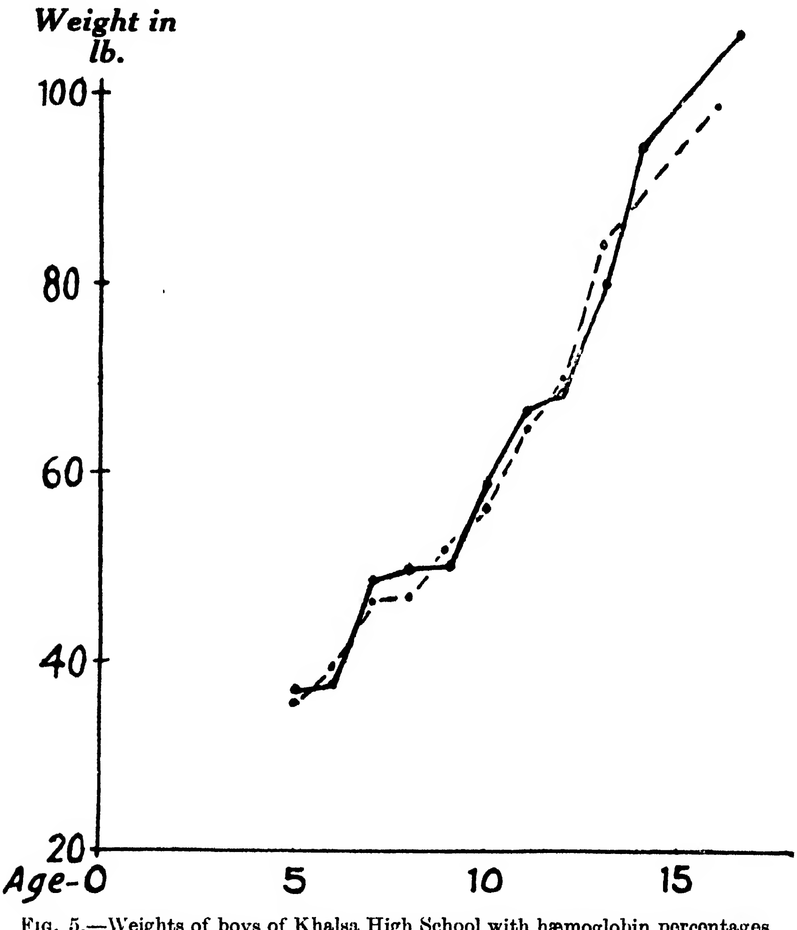

FIG. 5.—Weights of boys of Khalsa High School with hæmoglobin percentages above (continuous line) and below (broken line), respectively, the average of their own age groups.

in Figs. 2 and 5; the two weight curves of boys above and below the mean hæmoglobin of their own age groups, run parallel in the case of the Sikh boys, but diverge

in the case of the Bengalis; the boys with the hæmoglobin above normal are heavier in all but the two first age groups. The two height curves of the Sikhs also run nearly parallel (Fig. 6).

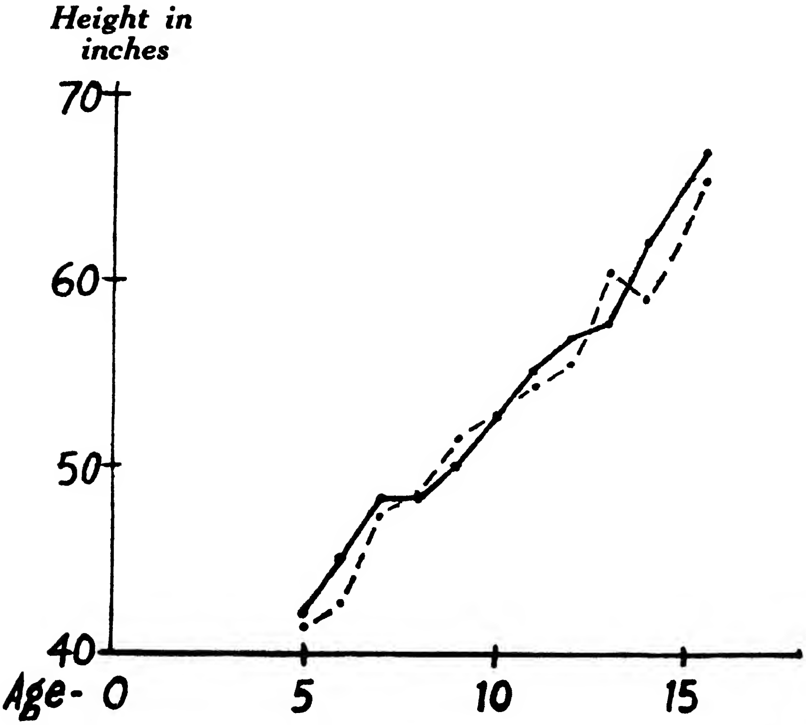

FIG. 6.—Height of boys of Khalsa High Schoo lwith hæmoglobin in percentages above (continuous line) and below (broken line), respectively, the average of their own age groups.

In the majority of the boys the systolic and diastolic pressures were also taken. Both rise steadily with the age. The correlation with hæmoglobin is shown in Table V. It is interesting that this is negative in the first few age groups and positive in all boys of 12 or above, but the correlation is not a marked one.

Table III.

Tirthapati Institution boys.

Age in years.	Number in group.	Mean in grammes per 100 c.c.	Height in feet and inches. Of all subjects. Range. Lowest.	Height: Of all subjects. Range. Highest.	Height: Of all subjects. N.	Height: Of all subjects. Mean.	Height: Of subjects with hæmoglobin above the mean. N.	Height: Above the mean. Mean.	Height: Of subjects with hæmoglobin below the mean. N.	Height: Below the mean. Mean.	Weight in pounds. Of all subjects. Range. Lowest.	Weight: Of all subjects. Range. Highest.	Weight: Of all subjects. N.	Weight: Of all subjects. Mean.	Weight: Of subjects with hæmoglobin above the mean. N.	Weight: Above the mean. Mean.	Weight: Of subjects with hæmoglobin below the mean. N.	Weight: Below the mean. Mean.	Correlation between hæmoglobin and height.	Correlation between hæmoglobin and weight.
8	63	12·11	3–7	4–10	61	4–2·52	30	4–2·27	31	4–2·77	32	77	59	50·98	29	49·48	30	52·4	–	–
9	61	12·06	3–11	4–11	61	4–4·52	31	4–4·87	30	4–4·17	44	79	57	53·95	30	53·49	27	54·41	+	–
10	65	11·89	4–0	5–6	64	4–6·06	32	4–6·44	32	4–5·69	44	103	63	58·49	31	61·0	32	57·95	+	+
11	90	12·46	4–2	5–5	89	4–11·02	38	4–9·95	51	4–7·65	46	112	89	65·45	38	74·82	51	62·92	+	+
12	90	12·41	4–3	5–8	89	4–11·04	38	5–0·84	51	4–9·71	46	126	89	75·53	38	81·52	51	71·24	+	+
13	72	12·59	4–3	5–8	71	5–1·39	32	5–3·13	39	4–11·97	57	119	71	82·16	32	85·25	39	79·64	+	+
14	55	13·10	4–10	5–9	55	5–2·83	27	5–4·15	28	5–1·57	65	141	55	94·69	27	102·52	28	83·15	+	+
15	34	13·30	4–9	5–9	34	5–4·38	18	5–5·67	16	5–2·93	73	175	34	98·51	18	106·50	16	89·56	+	+
16–18	23	13·39	5–3	5–10	23	5–5·74	12	5–6·08	11	5–5·36	87	152	23	105·70	12	111·58	11	103·64	+	+

TABLE IV.

Khalsa High School boys.

Age in years.	Number in group.	Mean in grammes per 100 c.c.	Height in feet and inches. Of all subjects. Range. Lowest.	Highest.	N.	Mean.	Of subjects with hæmoglobin. Above the mean. N.	Mean.	Below the mean. N.	Mean.	Weight in pounds. Of all subjects. Range. Lowest.	Highest.	N.	Mean.	Of all subjects with hæmoglobin. Above the mean. N.	Mean.	Below the mean. N.	Mean.	Correlation between hæmoglobin and height.	weight.
5	12	11·56	3–2	3–8	12	3–5·83	6	3–6·17	6	3–5·50	30	42	12	36·0	6	36·17	6	35·83	+	+
6	19	11·40	3–4	4–0	19	3–7·47	8	3–8·88	11	3–6·45	28	46	19	39·11	8	38·63	11	39·45	+	–
7	13	11·69	3–10	4–4	12	4–0	7	4–0·43	5	3–11·4	40	55	12	47·83	7	48·57	5	46·8	+	+
8	15	12·29	3–9	4–4	15	4–0·4	9	4–0·33	6	4–0·5	40	75	15	49·27	9	50·56	6	47·33	–	..
9	18	11·79	3–11	4–6	18	4–2·78	9	4–2·11	9	4–3·44	36	62	18	51·33	9	50·56	9	52·11	–	–
10	15	11·97	4–2	4–8	15	4–4·8	7	4–4·71	8	4–4·88	50	78	15	58·0	7	58·86	8	57·25	–	..
11	22	12·12	4–3	4–11	22	4–7·09	13	4–7·38	9	4–6·67	50	82	22	66·05	13	66·69	9	65·11	+	+
12	18	12·50	4–2	5–0	18	4–8·33	10	4–9·0	8	4–7·5	60	86	18	70·06	10	69·6	8	70·63	+	–
13	11	12·90	4–2	5–4	11	4–11·27	5	4–9·8	6	5–0·5	71	112	11	82·91	5	81·2	6	84·33	–	–
14	11	12·90	4–8	5–7	11	5–1·27	8	5–2·13	3	4–11·0	71	121	11	91·18	8	95·25	3	80·33	+	+
15–16	10	13·34	5–5	5–9	10	5–6·3	5	5–7·0	5	5–5·6	88	120	10	107·9	5	106·2	5	109·6	+	–

TABLE V.

Khalsa High School boys.

Age in years.	Number in group.	Mean in grammes per 100 c.c.	SYSTOLIC.								DIASTOLIC.								Correlation of hæmoglobin with	
			OF ALL SUBJECTS.				OF SUBJECTS WITH HÆMOGLOBIN.				OF ALL SUBJECTS.				OF SUBJECTS WITH HÆMOGLOBIN.					
			Range.				Above the mean.		Below the mean.		Range.				Above the mean.		Below the mean.			
			Lowest.	Highest.	N.	Mean.	N.	Mean.	N.	Mean.	Lowest.	Highest.	N.	Mean.	N.	Mean.	N.	Mean.	systolic.	diastolic.
8	15	12·29	80	110	10	97·3	6	95·5	4	100·0	40	80	10	60·5	6	60·83	4	60·0	−	+
9	18	11·79	80	120	12	99·42	6	93·0	6	105·83	45	80	12	62·75	6	58·0	6	67·5	−	−
10	15	11·97	80	115	14	99·21	7	97·86	7	100·57	40	80	14	62·72	7	62·14	7	63·29	−	−
11	22	12·12	88	125	20	103·7	12	102·08	8	106·13	55	95	20	71·45	12	71·58	8	71·25	−	+
12	18	12·50	90	160	17	110·65	10	115·3	7	104·0	55	90	17	73·0	10	74·8	7	71·43	+	+
13	11	12·90	100	132	10	112·31	4	115·0	6	110·5	55	100	10	78·2	4	78·75	6	77·83	+	+
14	11	12·90	100	128	11	114·82	8	116·63	3	110·0	60	98	11	82·55	8	82·88	3	81·67	+	+
15–16	10	13·34	110	130	9	121·33	4	121·5	5	121·2	28	100	9	82·89	4	90·75	5	76·6	+	+

HEIGHT, WEIGHT AND HÆMOGLOBIN CURVES IN BENGALIS AND SIKHS.

The hæmoglobin curves are contrasted in Fig. 4; it will be seen that the two curves run almost parallel. The two height curves are shown in Fig. 7;

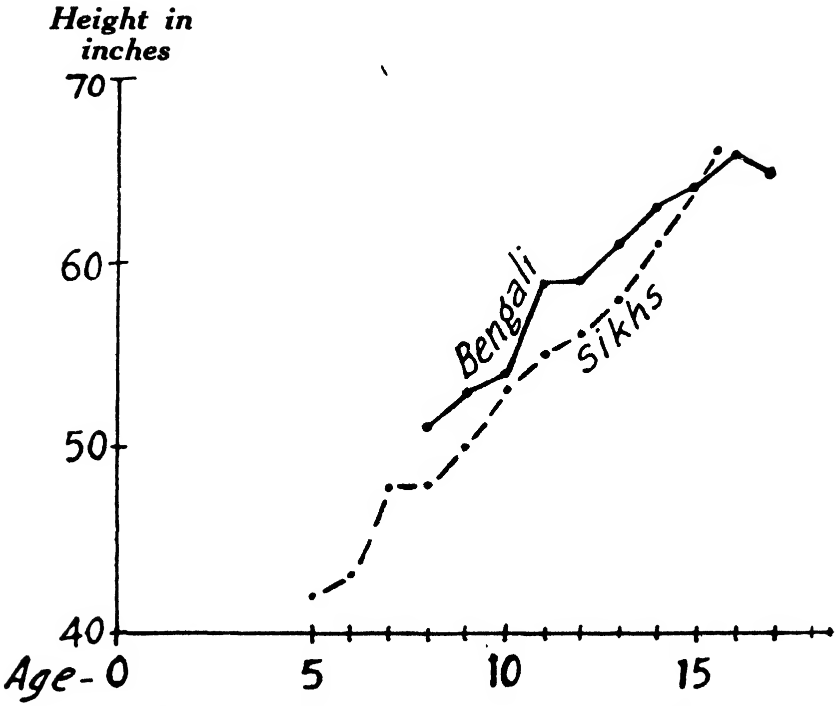

FIG. 7.—Heights at various ages, of Sikhs and Bengalis.

rather surprisingly, the Bengalis are taller, almost throughout, and only in the combined 15 to 16 years group are the Sikhs taller than the boys of the corresponding Bengali age group. The two weight curves run almost parallel, but the Sikh curve is again distinctly higher in the last age group (Fig. 8).

To summarize, the hæmoglobin curves in the two communities appear to be parallel; the weight curve is parallel in the earlier age groups but adolescent Sikhs are heavier; on the other hand Bengali children are taller up to the age of 14 after which the Sikhs are taller.

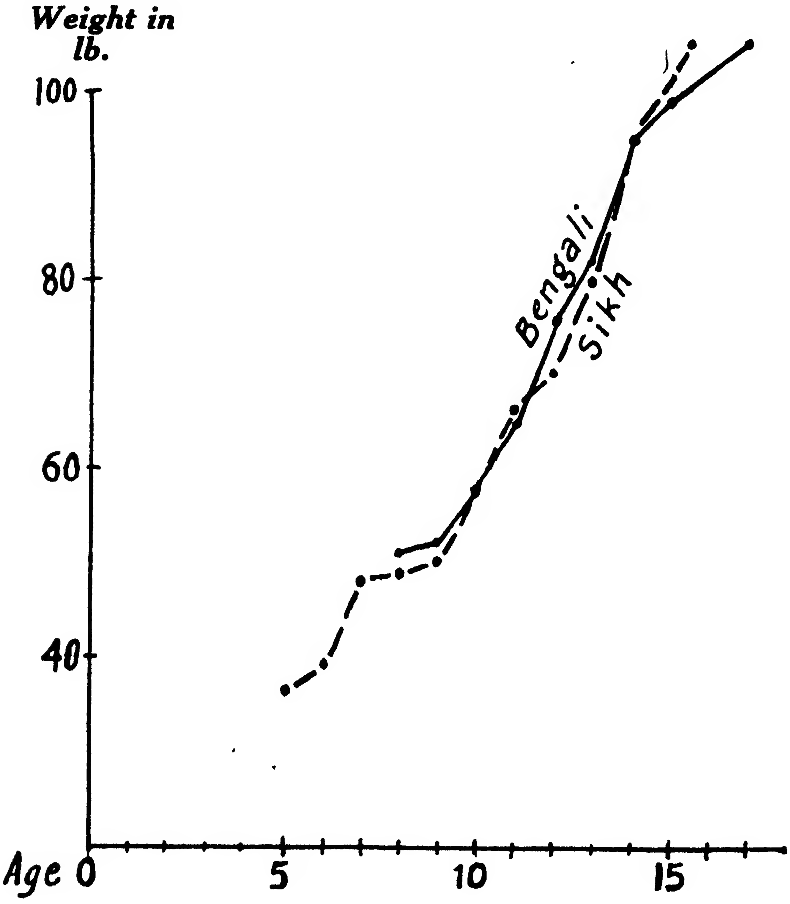

FIG. 8.—Weights at various ages, of Sikhs and Bengalis.

In Fig. 3, referred to above, it is shown that, if the Bengali boys are divided into two groups according to whether they are above or below the average weight of their age group, two significantly different hæmoglobin curves are produced, which are, respectively, above and below the hæmoglobin curve of the Sikh boys. The exact significance of this observation is not clear, but it seems to suggest

that it is possible to separate a group of well-nourished boys who have a higher hæmoglobin level than the rest, and conversely, that the hæmoglobin level of some of the remaining boys is not at its maximum and that this sub-maximal hæmoglobin is associated with under-nourishment.

Discussion.

The data on the hæmoglobin level of children and adolescents is as confused as was the normal hæmatological data for adults a few years ago. Nicholson and Cameron (1926) give figures from 13·7 grammes at the age of 5 years to 14·7 at $12\frac{1}{2}$ years, at which point the sexes diverge, the females not rising much higher. Fig. 9 shows a curve constructed from their paper. Haden's (1939) charts suggest

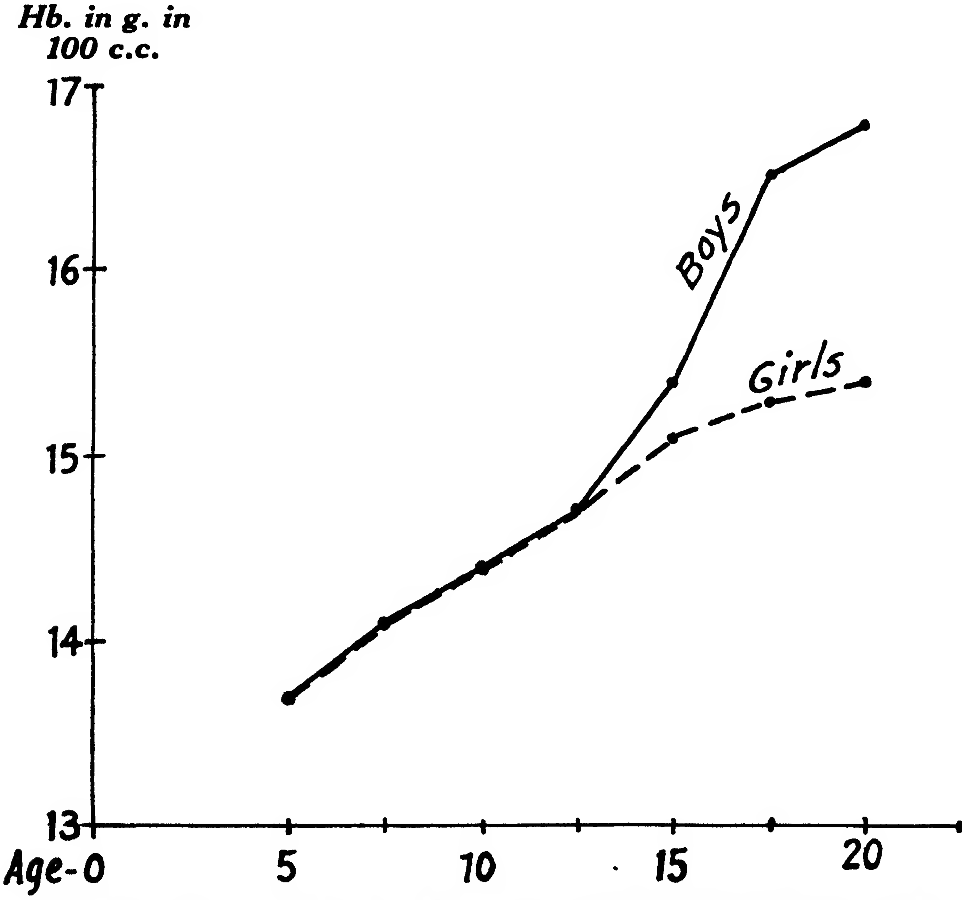

Fig. 9.—Hæmoglobin levels at various ages of boys and girls (United States) from figures given by Nicholson and Cameron (*loc. cit.*).

a higher range than this, about 14·4 grammes at the 5 to 6-year junction and 15·2 at the 10 to 11-year junction. On the other hand, Goldhamer and Fritzell (1933) give a number of figures for boys from 12 to 17 years; from these we have calculated the following means:—

Age group.	Number of boys.	Mean hæmoglobin in grammes.
12–13	8	11·03
13–14	33	11·21
14–15	17	11·55
15–16	27	11·84
16–18	15	12·31

These last figures are of an order entirely different from the others quoted above. There seems to be no explanation for this discrepancy regarding hæmatological data.

In our series, we have shown that, on the grounds of their being above or below the average weight of their age, it is possible to divide the Bengali boys into two groups in which the hæmoglobin curves are significantly different from one another; even in the better-nourished group, the highest point in the curve, at the 16-17-18-age group, is still under 14 grammes, whereas normal adults of the same class are at least 15 grammes. This seems to suggest that males do not reach the hæmoglobin level of the mature adult until the age of 25 years or so.

With females on the other hand the case is different. The male curve departs from the female curve at about 10 years and from this point, though the former still climbs sharply, the latter shows only a slight upward trend; the mean figure at 17 years is scarcely half a gramme above that of any of the 7 previous years, and the mean at 17 years, 12·18 grammes, is little short of that of non-pregnant adult females, aged 25 to 38, namely, 12·44 grammes (Napier and Das Gupta, 1940).

It is thus evident that, when considering the normal hæmoglobin values of male children and adolescents, an allowance must be made for age, but that, in the case of female children, it is less important, as their blood approximates to the adult level at a much earlier date.

The question of the diet of the two groups of boys has been gone into, but no accurate data are available, as the boys of both groups fed at their own homes. The

Sikh diet is generally considered to be the best diet in India, but inquiry does not suggest that the diet of these Sikhs living in Calcutta is much better than. or even as good as, the Bengali diet. They do take milk when they can afford it, but the milk and ghee in Calcutta are comparatively expensive and usually of poor quality. They take *chapattis* made of wheat flour, vegetables, a very little dâl, but no fish or meat. In their own country, they make up their calories by taking milk in larger quantities and by using ghee (clarified butter) in preparing their food.

The Bengali boys live on a staple diet of rice, but they take more dâl, also fish, and in some instances meat occasionally. On the whole, the social status of the Bengali boys was distinctly higher and their diet probably better. This explains why the Sikh boys, members of a physically superior race, show little evidence of this physical superiority.

Acknowledgments.

Our thanks are due to Captain J. S. Chowhan who made the arrangements for the investigations in the Khalsa High School and took the blood pressures and to the Superintendents of the Khalsa High School, of the Tirthapati Institution, and of the London Mission School, for their co-operation.

For assistance with collection of specimens, the estimation of hæmoglobin, etc., we must thank Drs. G. N. Sen, D. N. Majumdar, S. Mookherjee, and Misses Dei and Dharmvir, the last four workers under the Indian Research Fund Association.

Summary.

The hæmoglobin percentages of the blood, the heights and the weights of a number of Indian school boys and girls were taken.

The hæmoglobin curve of the boys showed a steady increase with the age from about 11·5 grammes at 5 years to about 13·5 grammes at 16 years. The latter figure is well below the mean hæmoglobin of adults of the same class, so that there is an indication that the hæmoglobin does not reach the adult level (about 15 grammes) until at least the 25th year.

The female curve departs from the male curve at about the 11th year and subsequently rises much more slowly to the female adult level (about 12·5 grammes).

There seems to be little difference between the hæmoglobin curve of the two groups of boys, Bengali and Sikhs, investigated.

The weight curves run parallel up to the 14th year after which the Sikh boys are heavier.

The Bengali boys are slightly taller up to 14 years of age after which the height curves cross.

If the natural trend is discounted there is no evidence of a constant correlation between either height or weight and hæmoglobin in the Sikh boys, but in the Bengali

boys the correlation of hæmoglobin with both height and weight is constantly positive after the age of 9 years. That is to say amongst the Bengalis there is a group of under-nourished boys whose hæmoglobin curve is distinctly and significantly lower than that of the better-nourished boys.

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WITHDRAWAL SYNDROME IN OPIUM ADDICTS AND THE RATIONALE OF TREATMENT WITH LECITHIN AND GLUCOSE.

BY

BREVET-COLONEL R. N. CHOPRA, C.I.E., M.A., M.D., SC.D. (Cantab.), F.R.C.P. (Lond.), I.M.S. (*Retd.*),

AND

CAPTAIN G. S. CHOPRA, M.B., B.S., A.I.R.O.

(*From the Department of Pharmacology, School of Tropical Medicine, Calcutta. Drug Addiction Inquiry, Indian Research Fund Association.*)

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INTRODUCTION.

THE present authors have made a careful study of the withdrawal symptoms in a series of 200 cases of opium addicts who were treated in the Carmichael Hospital for Tropical Diseases. All these individuals were males and a careful examination showed that they were not suffering from any other ailment of a serious nature before admission. In all cases the usual dose of opium was continued until the patients had become accustomed to their new surroundings and the hospital discipline. During this period the mentality of the patient with regard to the drug habit was carefully studied. It was made clear to him that in spite of all the treatments that he would receive, he may have to undergo considerable inconvenience and trouble from the symptoms which would follow the withdrawal of the drug, and that his active co-operation was absolutely essential at every stage of the treatment. This took from 2 to 5 days. After the patient had got quite used to the new surroundings, the supply of opium was suddenly and completely discontinued with the full knowledge and consent of the patient. The ensuing abstinence symptoms were carefully and systematically observed and noted in each case. The patients were put on lecithin and glucose treatment either on the day of withdrawal of opium or the day before.

Treatment with lecithin and glucose.

Apart from the above-mentioned series of 200 cases, this treatment has been tried with success on several thousand opium addicts who were taking doses of opium ranging from 45 to 250 grains daily in Upper Assam Valley during the recent prohibition campaign against the drug by the Assam Government. Briefly, the procedure adopted is as follows: Opium is suddenly withdrawn and a dose of 1 to 3 grains of calomel is given at bedtime which is followed by an effective dose of salts next morning. The last named is repeated every morning for the next few days to eliminate opium from the system generally and from the alimentary canal particularly and to stimulate the functions of the liver. On the day of withdrawal, or as soon as the symptoms appear, lecithin is given by mouth in doses of 15 to 20 grains thrice daily in form of pills. In a certain number of cases lecithin by this route produces nausea and, in such cases, the drug was given in the form of a colloidal solution by intramuscular injection, especially prepared for this purpose; the usual dose being 2 c.c. of 1 per cent solution twice daily. Experience has shown that lecithin when given by injection, acts just as effectively as when it is given by the mouth and in some cases even better. Besides this, the amount required to produce the desired effect is comparatively smaller and consequently the cost of treatment is considerably reduced. The administration of lecithin is continued till most of the major symptoms completely disappear, which takes 2 to 4 days. In severe cases it may be continued for a day or two after.

During the period of abstinence the patient is encouraged to take plenty of fluids and glucose by the mouth as these greatly ameliorate the symptoms of withdrawal. He is also given 25 c.c. of 25 per cent glucose along with 10 c.c. of 10 per cent calcium gluconate by the intravenous route during the first 5 days of the treatment. Glucose by the intravenous route helps in the elimination of opium alkaloids, stimulates the glycogenic function of the liver and in this way enables this organ to withstand the strain which undoubtedly falls on it. Glucose also serves as a ready food for the heart during the withdrawal period when all other food is generally refused. Calcium controls the muscular cramps which are commonly met with during the period of abstinence and is therefore added especially in cases which suffer severely from this symptom.

The duration of the treatment varies from 7 to 12 days according to the dose of opium taken, the duration of addiction and the age of the addict. In our hospital series the time of onset of different symptoms, the period of maximum intensity and the time of disappearance were recorded in each case. The effects of treatment on these symptoms were also noted.

Nervous and psychical disturbances.

A perusal of Table I will show the nature, frequency and course of the common nervous disturbances which were encountered in this series after complete and sudden withdrawal of the drug:—

TABLE I.

Shows the nervous and psychical disturbances in a series of 200 opium addicts on sudden withdrawal.

Symptoms.	Frequency, per cent.	Day of onset.	Days of maximum intensity.	Day on which they disappear.
1. Yawning, lassitude, general depression, and restlessness.	95	1st	3rd and 4th	14th
2. Headache	50	3rd	4th	7th
3. Cramps and pains in limbs ..	80	2nd	3rd	5th
4. Paræsthesias	15	3rd	5th	7th
5. Tremors of hands	6	3rd	5th	7th
6. Emotional disturbances, feeling of impending death, melancholia.	15	3rd	5th	10th
7. Insomnia and nervous excitability ..	80	1st	3rd to 5th	10th and after.

(1) The common symptoms met with were general malaise, mental depression, a feeling of being out of sorts, lassitude, yawning and heaviness in the head ; these were encountered in 95 per cent of this series. It may be said that these symptoms were not new to the addicts and were frequently experienced by them, in varying degrees of severity, during the interval between two doses and were only intensified on complete withdrawal of the drug. In most of the cases these symptoms set in during the first 24 hours and rarely later, i.e., from 2nd to 3rd or 4th day. As a rule, they lasted for a week or 10 days and then gradually disappeared under the effect of treatment towards the end of the second week. No special treatment was as a rule required for them, but in rare instances if they persisted beyond 10 to 12 days, a mixture containing iron, strychnine and arsenic was found to be helpful.

(2) *Headache.*—This was complained of in 50 per cent of the patients after withdrawal. This was at first mild, but at times, especially during the later stages of treatment, assumed an intense character. Slight headache which occurred 24 hours after the withdrawal of the drug was ascribed to sleeplessness. Very severe headache was met with in 4 cases in this series. In 2 patients it was felt as a sharp localized pain, while in the other 2 cases it was general and was so intense that the patients felt that the whole head was going to burst. This first appeared on the 4th day of the withdrawal and gradually disappeared on the 7th day after the administration of lecithin. For mild headache no special treatment was necessary but in severe cases such drugs as aspirin, veramon and other such analgesics were helpful.

(3) *Pains in the limbs and joints and cramps.*—These were very common and were recorded in 80 per cent of this series. In most of the cases actual pains were preceded by a feeling of lassitude and heaviness varying from a mere sensation of fatigue to intense pain in the joints and limbs. These pains become worse after 48 hours, and then gradually decline and practically disappear after the 5th day of the withdrawal. These generally yield to such simple measures as massage and hot baths, aspirin and barbiturates being sometime necessary.

Cramps in calf muscles recurring frequently at short intervals were met with in quite a number of cases. They appeared as a rule between the 2nd and 3rd day of withdrawal, attained their maximum on the 4th day and disappeared gradually on the 5th day. These were considerably relieved by administration of calcium gluconate, 10 c.c. of a 10 per cent solution of which was given along with glucose injections by the intravenous route. Local massage and copious drinks of glucose water by the mouth with administration of calcium lactate or gluconate was helpful in milder cases.

(4) *Parœsthesias.*—Immediately after the withdrawal of the drug, the skin especially of the face and the extensor surfaces of the limbs was flushed. The patients complained of a sensation of warmth in the chest and abdomen and sometimes in the legs. These symptoms were observed in 15 per cent in this series and attained their maximum intensity on the 3rd day, then declined and completely disappeared after 7 days.

A feeling of chill was also a fairly common symptom and was observed in 20 per cent in this series. There may also be a sensation of cold and shivers all over the body which may persist or may pass off quickly. If mild it requires no treatment, but when it persists calcium gluconate by the mouth or by injection is useful. Sometimes these symptoms are merely due to neurosis and in such cases a mixture containing bromides and valerian was found to be helpful.

(5) *Tremors of hands* were seen in 6 per cent of the patients on the 3rd to 5th day and disappeared after the first week. No special treatment was necessary as these are due to instability of the neuromuscular apparatus which soon becomes adjusted.

(6) *Psychical disturbances.*—Fifteen per cent of the cases in this series showed some psychical imbalance on the withdrawal of the drug. The inhibitory effect upon the higher centres is relaxed when the drug is suddenly stopped and therefore there is an uncontrolled flow of emotions. The patients exhibit different varieties of psychical disturbances according to their individual make-up and their nature and intensity vary according to the personality of the addict and the duration of the habit. The psychical disturbances were more pronounced in individuals who were taking large doses for prolonged periods and who suffered from nervous diathesis, than in those who were taking smaller doses for shorter periods and had no history of nervous diathesis. The common symptoms recorded were a feeling of general depression, sometimes a sensation of impending death, acute mental anguish, despair and even persistent melancholia. Restlessness was a very early symptom and was observed in almost all cases in varying degrees of intensity. It appeared on the 2nd day of the withdrawal, reached its maximum on the 4th day and then gradually disappeared. Depression was a comparatively later symptom and manifested itself on the 3rd to 5th day after the drug was stopped. It may be ushered in by a feeling of listlessness, mental dejection and melancholia. Treatment for this condition was on general lines the patient being encouraged and cheered as much as possible by the physician and the nursing staff. He should be assured that the symptoms were only a temporary phase and would pass off. In this series most of these symptoms entirely disappeared by the 10th day of withdrawal.

(7) *Insomnia.*—This was one of the earliest symptoms and appeared immediately after withdrawal. It attained its maximum intensity on 2nd to 5th day and sometimes continued for a few weeks or even months after the drug was withdrawn and treatment completed. No special treatment is necessary for this condition during the first few days except what is being done by way of administering lecithin and glucose. The patient at the same time is asked to exercise his own will power and try to sleep. If want of sleep is very distressing such simple measures as a hot foot-bath before retiring or a dose of bromide mixture at bedtime may be helpful. When these measures failed a pill containing 7½ grains of medinal administered at bedtime often succeeded in giving the patient sleep for 4 to 6 hours. This often produced a remarkable change in the patient's mental condition and attitude as he began to feel rested and gained confidence in the efficacy of the treatment. This drug, however, should not be repeated for more than 3 or 4 successive nights. Other barbiturates and hypnotics, such as adaline, evipan, luminal, ortal, were also tried with good results. We have also used small doses of 10 to 15 minims of tincture of *Rauwolfia serpentina* before bedtime with success. In one case of intractable insomnia where nothing succeeded, 20 minims of this tincture was successful in producing sleep and after a few days it could be stopped altogether.

Digestive disturbances.

The effects on the alimentary system are marked and severe when the patient is taking the drug in large doses. The results of analysis of various symptoms pointing to the derangement of the digestive organs have been summarized in Table II :—

Table II.

Shows the digestive disturbances produced in a series of 200 opium addicts on sudden withdrawal.

Symptoms.	Frequency, per cent.	Day of onset.	Days of maximum intensity.	Day on which they disappear.
1. Anorexia and distaste for food ..	80	2nd	4th to 5th	8th to 10th
2. Epigastric pain and abdominal disturbances.	18	3rd	4th to 6th	8th
3. Diarrhœa	45	2nd	3rd	5th
4. Nausea and vomiting	38	2nd	3rd	6th
5. General abdominal discomfort and pain.	18	4th	6th	14th

(1) *Anorexia and distaste for food.*—These are constantly present during the course of addiction, especially where the drug is being taken in large doses. These become more pronounced with the onset of withdrawal symptoms and were intense when the latter fully developed. They were observed in 80 per cent of cases. These symptoms reach their maximum intensity on 3rd to 5th day and disappear

by the end of 8th to 10th day. The patients are then able to take their normal diet.

(2) *Epigastric pain and abdominal disturbances.*—These were complained of by 18 per cent in this series, set in early and last long and are almost the last to disappear. They may be due to some intercurrent disease such as hookworm or chronic bowel infection for alleviation of which the habit was perhaps originally formed. They may, therefore, even persist after complete withdrawal and rehabilitation of the patient has taken place. The pathological condition underlying these symptoms should be properly diagnosed and treated.

(3) *Diarrhœa.*—Opium addicts are usually constipated during the period of addiction. During withdrawal, however, the peristaltic movements of the gut become more active and give rise to diarrhœa. Four to eight watery motions are of quite common occurrence in the early days of abstention. Diarrhœa is very often accompanied by a griping sensation in the abdomen and there may also be tenesmus. In the present series 45 per cent showed this symptom which appeared on the 2nd or 3rd day and disappeared on the 5th day. One of our patients suffered from a severe choleraic diarrhœa attended with all the signs and symptoms of collapse for which 6 pints of saline had to be given intravenously.

(4) *Nausea and vomiting.*—These were common symptoms and were observed in 38 per cent of this series during first 3 days and disappeared by the end of the first week. In 20 patients there was actual vomiting and in 2 cases it was severe and incessant. Sucking of ice greatly relieved these symptoms and in severe cases 10 drops of adrenaline hydrochloride 1 in 1,000 under the tongue every 2 to 4 hours gave relief. In 2 patients severe bilious vomiting occurred and the stomach had to be washed out with a solution of sodium bicarbonate (1 dram to a pint of water) after which the patient felt relieved.

Disturbances of body secretions.

General imbalance of secretions of the body is a very common phenomenon after withdrawal of opium. The symptoms observed under this heading have been given in Table III :—

Table III.

Shows the imbalance of general secretion of body as seen after withdrawal in 200 opium addicts.

Symptoms.	Frequency, per cent.	Day of onset.	Days of maximum intensity.	Day on which they disappear.
1. Running of the nose and sneezing ..	70	1st	3rd	5th
2. Watering of the eyes and lacrymation	50	Few hours	2nd	4th
3. Salivation	20	1st	3rd	6th
4. Sweating	10	2nd	3rd	7th

(1) *Running of the nose and sneezing.*—These were seen in 70 per cent of this series and appeared on the 1st day and attained their maximum intensity on the 3rd day. These symptoms disappeared on the 5th day in 70 per cent of the cases and in the remainder they persisted even after the complete disappearance of all other symptoms of withdrawal. An examination of the nasal cavities and accessory sinuses in such cases always revealed some pathological condition for which the habit was started. Radical treatment by way of operation and general treatment was recommended in such cases.

(2) *Running of the eyes and lacrymation.*—These were observed in over 50 per cent of this series and appeared within a few hours after withdrawal. Maximum intensity was observed on the 2nd day and on the 4th day they disappeared except in cases who were suffering from some eye affections such as trachoma, blepharitis, trachiasis, etc. In such cases suitable treatment of the particular condition is needed.

(3) *Salivation.*—This was seen in 20 per cent of this series and disappeared by the end of the 6th day.

(4) *Sweating.*—Perspiration accompanied by yawning and general lassitude were observed in 10 per cent of the cases during early stages of withdrawal. Sweating was very troublesome to the patient and difficult to control. It generally appeared on the 1st day of withdrawal and disappeared by the end of the first week.

(5) *Spermatorrhœa.*—In this series 5 per cent of the patients suffered from this complaint frequently on the 5th to 7th day of withdrawal. No special treatment was adopted except a dose of salts every morning and mixture which contained bromides.

Cardiovascular system.

In patients in this series electrocardiographic examinations were made before withdrawal, during withdrawal and after treatment. None of them showed any remarkable abnormality except in one case in which signs of symptoms of serious heart block were observed on withdrawing of the drug.

Summary and discussions.

From the foregoing analysis of withdrawal symptoms as observed in a series of 200 hospital cases and from our previous studies (Chopra and Chopra, 1933, 1935) it would appear that while the whole body is affected more or less the central nervous system and the digestive system bear the brunt of this addiction, and when the drug is withheld there is a general explosion of symptoms pertaining to these systems particularly. In addiction of long duration, general dehydration of the body takes place owing to excessive perspiration, abnormal salivation and obstinate diarrhœa. These observations have also been made by such workers as Sollier (1910), Maguin (1909), Pierce and Plant (1928) and others. These facts, however, strongly suggested to us that such a dehydration process may have an ultimate effect on the fluid content of the blood. From our observation that in most of the cases the state of addiction was invariably accompanied by an

increase in the fluid content of the blood, we were inclined to believe that an excessive secretion on withdrawal will have a reverse effect leading to an increase in the percentage of serum proteins. In the literature, however, we find several workers have observed an increased hydræmic state of the blood on withdrawal leading to a further dilution as compared to pre-withdrawal period. Thus, Barbour, Hunter and Richey (1929) are of opinion that a 'hypersecretion of morphine withdrawal rather than causing a hypothetical dehydration or "detoxification" may be looked upon as a natural accompaniment of the hydration of blood and probably of the tissues in general'. They, however, explain their conclusions on the hypothesis that 'regardless of diet, addiction diminishes the water intake as well as the water "deficit". Withdrawal evokes a decided overcompensation in both the above factors'. Pierce and Plant (*loc. cit.*) also subscribe to a similar opinion.

The recent work of Chopra and Roy (1937) on the blood-lipoid changes in opium addicts before, during and after withdrawal, on the contrary, pointed to the probability of a diminution in the water content of the blood on withdrawal. This led Chopra and Ganguly (1939) to actually determine the effect of withdrawal on the blood fluid and also to explain, if possible, the rationale of the treatment of opium addiction with lecithin and glucose so successful in our hands. In the above series the urine of the addicts under treatment was regularly examined daily for the presence of morphine to make sure that the subject was not surreptitiously taking the drug. Blood sera from these addicts were examined for total proteins before, during and after the withdrawal of the drug. In most of the cases it was found that concentration of proteins present in the serum before treatment underwent a definite increase during the withdrawal period and returned gradually to almost the pre-withdrawal value after the addict had undergone the prescribed treatment. In very few instances, however, the percentage of protein during the withdrawal period instead of showing an increase was found to have diminished. This observation suggested that there might be some other factors responsible for controlling the total protein content of the blood.

The increase in the total protein content during the period of withdrawal as a general rule runs parallel to the appearance of withdrawal symptoms. Excessive outflow of water from the body is one of the marked withdrawal symptoms that has been observed in almost all the cases studied by us. This apparently points to a disturbance in the fluid equilibrium in general in the body. The drainage of fluid is likely to affect the blood in the long run and the loss of water that the blood has to suffer due to an excessive drainage may increase the percentage of serum proteins. The concentration of other blood constituents may also be affected in a similar way.

From the data gathered after treatment it was evident that the proteins in the majority of cases return almost to their original values. The effect of treatment may, therefore, be taken to have restored the fluid equilibrium of the system to its previous level. In a previous paper by Chopra, Mukherjee and Chopra (1935) it was indicated that the observed increase of euglobulin probably meant an ultimate drainage of phosphate from the nerve cells. Lecithin treatment was, therefore, suggested on that basis. In the majority of cases lecithin decreased the intensity

of withdrawal symptoms and shortened their duration. But in spite of its administration, the abstinence symptoms were very severe in some of the subjects and in these cases intravenous injections of 25 c.c. of 25 per cent glucose helped to ameliorate the condition. Although lecithin was unable to cope with the severity of the withdrawal symptoms it doubtless removed the craving for the drug in the majority of cases.

However, the rôle of glucose in such cases can be understood from our present observations. By treatment the ultimate effect seems to be the restoration of the water balance. Therefore, any drug that confers a fluid retaining power to the blood is expected to have a good effect. Carbohydrates in general and glucose in particular are known to possess this water-retention capacity. Glucose, therefore, in addition to stocking the liver with glycogen to enable it to cope with the unusual strain on this organ during the process of elimination of morphine, helps the retention of water in the blood and keeps up the blood hydration level to its normal value. From the above we may, therefore, conclude that lecithin tones up the nerves of the addicts and glucose helps to restore the disturbed water balance. Thus, it is not difficult to see how these two together produce the desired effect in removing the drug habit and alleviating abstinence symptoms in an opium addict.

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THE ANTI-HÆMOLYTIC ACTION OF 'SOLUSEPTASINE': A DRUG BELONGING TO THE SULPHANILAMIDE GROUP.

BY

A. C. ROY, M.SC.,

D. C. MAZUMDAR, M.B., M.R.C.P. (Lond.),

AND

P. MUKHERJEE, B.SC.

(From the School of Tropical Medicine, Calcutta.)

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Reports on the therapeutic trials of the drugs of the sulphanilamide group from different quarters and also results from our own trial in a large number of cases of pneumonia, cerebrospinal meningitis, gonorrhœa and streptococcal infections, have both been very encouraging. These drugs have indeed proved themselves to be very formidable weapons in combating diseases originating with coccal infections and record a distinct triumph in chemotherapy more far reaching in their application than any other drug hitherto discovered.

In vitro experiments have not yet been able to furnish any information which might enable us to assess the curative action of the sulphanilamide drugs. Their mode of action is still obscure. Far more powerfully antiseptic substances exist which have no action on general coccal infection. The present position of chemotherapy by drugs of the sulphanilamide group has been very ably reviewed by Browning (1939). For some time past, one of us (A. C. R.) has been studying the hæmolytic properties of various substances, such as snake venoms, bile salts, saponins, soaps and the different agents which either retard or accelerate this phenomenon in different cases. We thought it would be interesting to see if the sulphanilamides have any action on any of these hæmolytic substances and on cobra venom in particular.

As Prontosil Soluble (Bayer) itself has a deep red colour, it could not for obvious reasons be employed for hæmolytic experiments. Of the two other colourless

compounds tried sulphanilamide (Crookes) was not found to be soluble in normal saline to any appreciable extent and we did all our experiments with Soluseptasine (May and Baker) which is disodium para (γ-phenylpropylamino) benzene sulphonamide-a, γ-di-sulphonate.

The experiments were done as far as possible under aseptic conditions. A 0·05 per cent solution of cobra venom in normal saline and a 5 per cent suspension of human r.b.c. were used, the total volume being 1·0 c.c. in every case. Cobra venom and Soluseptasine were mixed together and incubated for half an hour at 37°C. before r.b.c. suspension was added. Readings were taken at stated intervals up to 2 hours at 37°C. when the tubes were kept inside the ice-chest over-night and readings were again taken on the following day.

TABLE I.

Effect on cobra venom hæmolysis.

	R.b.c., c.c.	Venom solution, c.c.	Normal saline, c.c.	Soluseptasine 10 per cent solution, c.c.	HÆMOLYSIS. ½ hour.	1½ hours.	21 hours.
1	0·3	0·5	0·2	—	++ +++	..	..
2	0·3	0·5	—	0·2	—	—	+++ ++
3	0·3	0·2	0·5	—	+++++	..	..
4	0·3	0·2	0·3	0·2	—	—	About 2 per cent hæmolysis.
5	0·3	0·1	0·6	—	5 per cent.	+++++	..
6	0·3	0·1	0·4	0·2	—	—	—
7	0·3	0·05	0·65	—	—	—	—
8	0·3	0·05	0·45	0·2	—	—	—
9	0·3	—	0·5	0·2	—	—	—
10	0·3	—	0·7	—	—	—	—

These results show that Soluseptasine has undoubtedly a retarding action on cobra venom hæmolysis. This experiment was repeated several times with

almost identical results. This action of Soluseptasine on cobra venom hæmolysis is brought out in a more striking manner in the following experiments (Table II) when varying quantities of both cobra venom and Soluseptasine were used.

TABLE II.

	R.b.c., c.c.	Venom solution, c.c.	Normal saline, c.c.	Soluseptasine 10 per cent, c.c.	Hæmolysis.			
					½ hour.	1 hour.	2 hours.	21 hours.
1	0·3	0·5	-	0·2	—	—	—	+++++
2	0·3	0·5	0·1	0·1	—	—	+++++	..
3	0·3	0·5	0·15	0·05	—	+++++	..	..
4	0·3	0·5	0·2	—	+++++	..	..	..
5	0·3	0·4	0·1	0·2	—	—	—	10 per cent.
6	0·3	0·4	0·2	0·1	—	—	+++++	..
7	0·3	0·4	0·25	0·05	—	—	+++++	..
8	0·3	0·4	0·3	—	+++++	..	..	..
9	0·3	0·3	0·2	0·2	—	—	—	10 per cent.
10	0·3	0·3	0·3	0·1	—	—	—	+++++
11	0·3	0·3	0·35	0·05	—	+++++	..	..
12	0·3	0·3	0·4	—	+++++	..	..	..
13	0·3	0·2	0·3	0·2	—	—	—	±
14	0·3	0·2	0·4	0·1	—	—	+++++	..
15	0·3	0·2	0·45	0·05	-	+++++	..	..
16	0·3	0·2	0·5	—	+++++	..	..	..
17	0·3	0·1	0·4	0·2	-	—	—	—
18	0·3	0·1	0·5	0·1	—	—	—	20 per cent.
19	0·3	0·1	0·55	0·05	—	—	—	+++++
20	0·3	0·1	0·6	—	90 per cent.	+++++	..	..
21	0·3	—	0·5	0·2	—	—	—	—
22	0·3	-	0·6	0·1	—	—	—	—
23	0·3	—	0·65	0·05	—	—	—	—
24	0·3	—	0·7	—	—	—	—	—

It will be seen from Table II that the strength of the venom remaining the same the extent of retardation is roughly proportional to the amount of Soluseptasine added. We are carrying on experiments with a view to see whether this drug can neutralize any of the other toxic principles of the venoms and the results will be reported in due course.

With a view to see what action, if any, this drug has upon some of the other well-known hæmolytic substances, experiments were done on similar lines with the saponins, bile salts, soaps, and also bacterial hæmolysins with the following results :—

TABLE III.

Effect on sodium glycocholate (Merck's) hæmolysis.

	R.b.c., c.c.	Na-glycocholate 1 in 1,000. c.c.	Normal saline, c.c.	Soluseptasine 5 per cent solution, c.c.	Hæmolysis.
1	0·3	0·5	0·2	--	Complete in 2 mins. 30 sec.
2	0·3	0·5	-	0·2	,, 6 ,, 55 ,,
3	0·3	0·4	0·3	—	,, 4 ,, 5 ,,
4	0·3	0·4	0·1	0·2	,, 24 ,, 0 ,,
5	0·3	0·3	0·4	—	,, 12 ,, 26 ,,
6	0·3	0·3	0·2	0·2	,, 96 ,, 0 ,,
7	0·3	0·2	0·5	—	95 per cent hæmolysis in 96 mins.
8	0·3	0·2	0·3	0·2	No hæmolysis in 21 hours.
9	0·3	0·1	0·6	—	Do.
10	0·3	0·1	0·4	0·2	Do.

TABLE IV.

Effect on sodium taurocholate (DIFCO) hæmolysis.

	R.b.c., c.c.	Taurocholate 1 in 500, c.c.	Soluseptasine 5 per cent solution, c.c.	Normal saline, c.c.	Hæmolysis.
1	0·3	0·5	—	0·2	Complete in 2 mins. 0 sec.
2	0·3	0·5	0·2	—	,, 2 ,, 0 ,,
3	0·3	0·4	—	0·3	,, 3 ,, 10 ,,
4	0·3	0·4	0·2	0·1	,, 7 ,, 30 ,,
5	0·3	0·3	—	0·4	,, 8 ,, 0 ,,
6	0·3	0·3	0·2	0·2	80 per cent in ½ hr. } Complete in 21 hrs.
7	0·3	0·2	—	0·5	40 ,, ½ hr. }
8	0·3	0·2	0·2	0·3	10 ,, 21 hrs.
9	0·3	0·1	—	0·6	2 ,, 21 ,,
10	0·3	0·1	0·2	0·4	No hæmolysis in 21 hrs.

In the above experiments all the other components were mixed and incubated at 37°C. for half an hour before the addition of r.b.c. The results given in Tables III and IV show that Soluseptasine also retards bile salt hæmolysis excepting in those cases where the concentrations of the bile salts were high.

Similar results were obtained with saponin (Merck's) and cyclamine (Merck's) but this retardation was evident only in relatively higher dilutions of the substances when the rate of hæmolysis without Soluseptasine was comparatively slow.

The effect of this drug upon sodium oleate (Merck's) was also studied but no clear-cut results could be obtained as the drug was found to behave in an erratic manner sometimes accelerating and at others retarding hæmolysis caused by sodium oleate.

Bacterial hæmolysins.

Under this head cholera hæmolysin and streptococcal hæmolysins were studied. Cholera vibrio (El Tor 46) were grown in 1 per cent peptone solution and *Streptococcus hæmolyticus* in serum broth. In both the cases 18-hour-old cultures were centrifuged and the supernatant fluid was used for hæmolytic experiments. It may be mentioned in this connection that though the bulk of the bacterial bodies were thrown down by centrifugalization, the supernatant fluid was not absolutely free from these organisms.

Table V.

Effect on cholera hæmolysin.

	R.b.c., c.c.	Hæmolysin, c.c.	Normal saline, c.c.	Soluseptasine 10 per cent, c.c.	Hæmolysis.			
					½ hour.	1 hour.	1½ hours.	21 hours.
1	0·3	0·3	0·4	—	—	·	+ +	+ + + + + +
2	0·3	0·3	0·2	0·2	—	—	—	—
3	0·3	0·2	0·5	—	—	—	—	95 per cent.
4	0·3	0·2	0·3	0·2	—	—	—	—
5	0·3	0·1	0·6	—	—	—	—	95 per cent.
6	0·3	0·1	0·4	0·2	—	—	—	—
7	0·3	0·05	0·65	—	—	—	—	95 per cent.
8	0·3	0·05	0·45	0·2	—	—	—	—
9	0·3	—	0·7	—	—	—	—	—
10	0·3	—	0·5	0·2	—	—	—	—

TABLE VI.

Effect on streptococcal hæmolysin.

	R.b.c., c.c.	Hæmolysin, c.c.	Normal saline, c.c.	Soluseptasine 10 per cent, c.c.	Hæmolysis.				
					½ hour.	1 hour.	1½ hours.	21 hours.	Additional incubation for 3 hours at 37°C.
1	0·3	0·3	0·4	—	—	—	—	90 per cent.	++++++
2	0·3	0·3	0·2	0·2	—	—	—	—	—
3	0·3	0·2	0·5	—	—	—	—	90 per cent.	++++++
4	0·3	0·2	0·3	0·2	—	—	—	—	—
5	0·3	0·1	0·6	—	—	—	—	20 per cent.	++++++
6	0·3	0·1	0·4	0·2	—	—	—	—	—
7	0·3	0·05	0·65	—	—	—	—	5 per cent.	10 per cent.
8	0·3	0·05	0·45	0·2	—	—	—	—	—

++++++ Complete hæmolysis.
± Doubtful ,,
— No hæmolysis.

The results given in Tables V and VI show that Soluseptasine neutralizes the hæmolysins elaborated by *Vibrio choleræ* and *Streptococcus hæmolyticus*.

SUMMARY AND CONCLUSIONS.

1. The effect of Soluseptasine, a drug belonging to the sulphanilamide group, on some of the common hæmolytic substances was studied.

2. It was found to have a retarding action on hæmolysis caused by cobra venom, bile salts, saponin and cyclamin.

3. Bacterial hæmolysins such as those derived from *Vibrio choleræ* (El Tor) and *Streptococcus hæmolyticus* are also neutralized by Soluseptasine.

4. Its action on sodium oleate hæmolysis was irregular.

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INHIBITORY AGENTS OF UTERINE MOTILITY*.

BY

B. T. KRISHNAN.

Central Institute of Physiology, Madras Medical College, Madras.

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ALTHOUGH the inhibitory influence of the hormone of corpus luteum on uterine motility has been established in various animals and in women by the work of Knaus (1926, 1930), Robson and Illingworth (1931), Reynolds (1936) and others, it has been observed by Robson (1935) and Reynolds (1937) and also in a few of my experiments that, in certain species as the rat, mouse, and guinea-pig, the uteri excised during pseudo and true pregnancy show rhythmical contractions and respond to pituitrin. Castration in early or mid-pregnancy does not interfere with the pregnancy or parturition in the cat, guinea-pig, horse, and donkey (Snyder, 1938). Numerous instances of castration in women in early pregnancy without causing abortion have been reported. Post-coital uterine quiescence has been observed by Reynolds and Friedman (1930) and others in the rabbit, ferret, and cat before ovulation and the development of the corpus luteum.

These observations indicate that there are agents in the body other than the corpus luteum hormone which exert an inhibitory influence on the rhythmical contractions and the pituitrin reaction of the uterus. Under experimental conditions, even the corpus luteum hormone has no direct immediate effect on uterine motility *in vivo* or *in vitro*. It is only after a series of subcutaneous or intramuscular injections of the hormone into non-pregnant normal or ovariectomized animals that any inhibitory effect is observed. The inhibition caused by the luteal hormone is apparently the result of its prolonged action on the uterine muscle either directly or through other hormonic or chemical agencies in the body.

In this paper are embodied the results of experiments carried out on 24 guinea-pigs, 12 cats, and 6 rats, pregnant and non-pregnant, normal and ovariectomized, with a view to find out the possible inhibitory agents in the body other than the luteal hormone.

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1. A. P. L. principle in pregnancy urine.

Reynolds and Friedman (1930) found in rabbits a decrease in the uterine activity on injection of urine from a pregnant woman. Later in 1932 they reported that there was a temporary diminution of uterine motility 3 to 6 hours after the urine substance, in subliminal doses for ovulation, was injected into rabbits (Reynolds and Friedman, 1932). They found similar results in 6 out of 10 œstrin injected castrated rabbits. Robson (1933) demonstrated that pregnancy could be prolonged to 40th day in rabbits by injecting pregnancy urine extracts.

In my experiments on rats, guinea-pigs, and cats, antuitrin S (P. D. & Co.) in doses of 0·1 c.c. to 0·5 c.c. (10 to 50 rat units) caused no inhibition of the rhythmical contractions or the pituitrin reactions of the excised uteri in bath. On injection into an anæsthetized pregnant cat, it had no inhibitory effect on the uterus whose contractions were recorded *in vivo.* The results were also negative when ovariectomized guinea-pigs were given daily injections of 50 rat units (0·5 c.c.) of antuitrin S for 10 days and the uterine contractions were recorded *in vivo* under anæsthesia and also *in vitro,* 4 to 5 hours after the last injection. These results show that antuitrin S or A. P. L. principle does not serve as an inhibitor of uterine motility in guinea-pigs, cats, and rats.

2. Anterior pituitary hormone.

Reynolds and Friedman (1930) suggested that the post-coital uterine quiescence of the uterus in the rabbit may be due to humoral changes responsible for ovulation. Marshall and Verney (1936) found that stimulation of the brain or lumbar spinal cord brought about ovulation after a delay of 17 to 24 hours and suggested that the effect of central nervous stimulation was liberation of the anterior pituitary hormone which is responsible for ovulation. In the case of rabbit, ferret, and cat, possibly the orgasm on coitus causes through nervous excitation liberation of the anterior pituitary hormone which may be responsible not only for ovulation but also for the uterine quiescence which occurs before ovulation. Such inhibitory action, if present, cannot be peculiar to rabbit, ferret, and cat but must be observable in other animals as well.

With a view to find out the direct action, if any, of the anterior pituitary on the uterus, antuitrin (P. D. & Co.), an extract of the whole anterior lobe of the pituitary, was injected every alternate day into ovariectomized guinea-pigs, intramuscularly in doses of 0·5 c.c. After a fortnight, 4 to 5 hours after the last dose, the uterus was excised and placed in a bath of warm oxygenated Ringer-Locke's fluid. The contractions recorded did not show that the uterus was under any inhibitory influence in the body. Direct addition of antuitrin to the bath had no effect on the uterine movements. On intravenous injection of antuitrin (0·5 c.c.) into an anæsthetized non-pregnant cat, there was a slight rise in the tone of the uterus whose contractions were recorded *in vivo.* Antuitrin G (P. D. & Co.), substantially free from the thyreotropic and gonadotropic principles, caused a strong contraction of the cat's and guinea-pig's uterus *in vitro* as in Plate IV, fig. 1. These results

show that the anterior pituitary extract has no inhibitory influence on the uterine contractions in guinea-pigs and cats, but on the other hand is excitatory.

3. Thyroxine.

As the activity of the thyroid gland is closely related to the sexual cycle in the female, the thyroid hormone may possibly have a direct or indirect influence on the uterine motility. Toth (1928) observed that prolonged feeding with large doses of thyroid increased the frequency and altered the character of rhythmical contractions of the guinea-pig's uterus but thyroxine when injected did not produce such results. *In vitro*, there was diminution of tone on addition of thyroxine to the bath.

In my experiments, on injecting thyroxine sodium (0·1 mg.) daily intramuscularly into non-pregnant normal and ovariectomized guinea-pigs for 20 days and placing the excised uteri on the last day in a warm bath of oxygenated Ringer-Locke's fluid, normal rhythmical contractions and pituitrin reactions were observed. Direct addition of thyroxine sodium to the bath had no effect. Thyroxine may, therefore, be considered to have no inhibitory influence on uterine motility in guinea-pigs.

4. Adrenaline.

The action of adrenaline on the uterine muscle is said to vary in different species of animals and according to the pregnant or non-pregnant condition of the uterus. In pregnancy it is supposed to be generally augmentory. But in a few of my experiments on rats and guinea-pigs, adrenaline was found, *in vitro*, to be inhibitory both in the case of the pregnant and the non-pregnant uterus. It may, therefore, be said that, in such of the animals in which it acts as an inhibitory agent on the pregnant uterus, any excess of adrenaline secreted in the body during pregnancy will have an influence on uterine motility.

5. Insulin.

Tested *in vitro* insulin was found to have no direct influence on the uterus of the guinea-pig, pregnant or non-pregnant, but it was observed that, on addition of pituitrin to the bath after insulin, the pituitrin reaction of the pregnant as well as of the non-pregnant uterus was intensified as in Plate IV, fig. 2.

6. Vitamin E.

Beneficial results have been reported clinically in America and England by oral administration of wheat germ oil in cases of habitual and threatened abortion (Watson and McArthur, 1937). Vitamin E is said to promote fœtal development and keep up the placental circulation intact and thus prevent abortion. The true physiological rôle of vitamin E is yet to be known. Beneficial results in cases of abortion are also given by the administration of corpus luteum extracts. Vitamin E may have an influence on the growth of luteal tissue either directly or through the anterior pituitary. Rowlands and Singer (1936) showed that the luteinizing

activity of the pituitary in vitamin-E deficient rats is about half that of normal rats. Barrie in 1937 described degeneration of acidophiles and basophiles of the anterior pituitary and in 1938 reported fatty degeneration of uterine muscle fibres, pigmentation, and development of fibro-myomata in vitamin-E deficiency (Barrie, 1937, 1938).

With a view to find out the influence, if any, of vitamin E on uterine motility wheat germ oil, in 6 minim doses, was injected daily intramuscularly or intraperitoneally into non-pregnant normal and ovariectomized guinea-pigs for 22 to 27 days and, at the end of this period, the excised uterus of each animal was tested *in vitro*. The excised uterus of the ovariectomized guinea-pig was found to be much more sensitive to pituitrin than the one from the normal animal. The uterus of one normal animal, which had developed slight peritonitis after the intraperitoneal injections of wheat germ oil, was sluggish in its reaction to pituitrin at first, reacting only to 0·1 c.c. and higher doses, but after repeated washings the reactions improved and even 0·01 c.c. of pituitrin was effective. The sluggish behaviour at the beginning was probably due to the effects of peritonitis. The ovaries removed after the series of injections of wheat germ oil and examined histologically showed a fairly higher follicular development and a greater formation of luteal tissue than the normal ones. From these observations it is concluded that vitamin E has no direct action on the uterine muscle but may exert an inhibitory control on uterine motility by influencing the growth of luteal tissue.

7. Other chemical agents.

While experimenting with the corpus luteum extracts produced by various manufacturers, it was observed that the soluble extract of corpus luteum (P. D. & Co.) had a stimulating effect on the pregnant as well as on the non-pregnant uterus of the guinea-pig *in vitro* as in Plate IV, figs. 3*a* and 3*b*. As this extract is said to contain 0·5 per cent chloretone, chloretone alone of the same strength was added to the bath after washing the excised uterus. The rhythmical movements were unaffected but the pituitrin contraction was inhibited. The stimulating effect of the P. D. & Co's extract is, therefore, considered to be due to some other ingredient used in the preparation of the soluble extract.

The watery extract produced by the Bengal Immunity Co. for oral administration caused, on the other hand, a profound inhibition of the rhythmical movements and the pituitrin reaction as in Plate IV, figs. 4*a* and 4*b*. The inhibitory effect was apparently due to the chemical agents used in the preparation of the extract. As the extract smelt of thymol, 0·5 c.c. of thymol in glycerine (1 in 200) was added to the bath while the excised uterus of a guinea-pig was reacting to pituitrin. There was immediate inhibition of the pituitrin contraction as in Plate IV, fig. 5. Thymol in watery solution (1 in 1,500) had a similar relaxing effect. When an excised guinea-pig's uterus, which was very sensitive even to a dose of 0·0001 c.c. of pituitrin, was in the bath and when pituitrin up to 0·001 c.c. was added after thymol, there was no reaction. When a stronger dose of 0·01 c.c. of pituitrin was added, there was a feeble contraction as in Plate IV, fig. 6. Intravenous injection of

Plate IV.


Fig. 1.

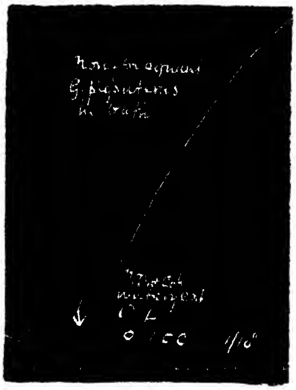
Fig. 3b.

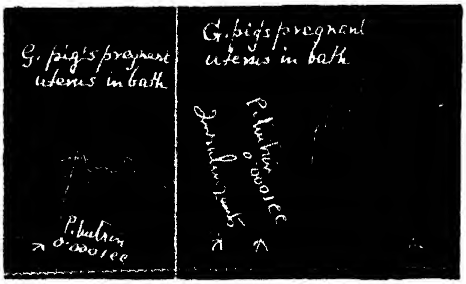

Fig. 2.

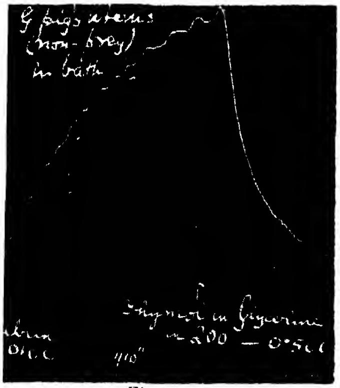

Fig. 5.

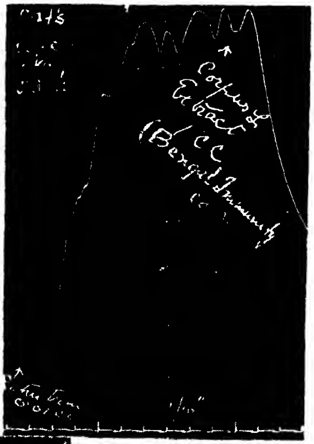

Fig. 4a.

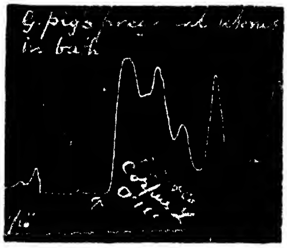

Fig. 3a.

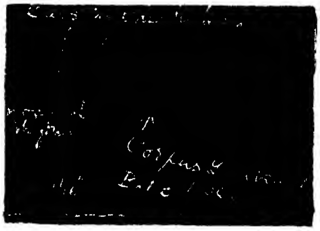
Fig. 4b.


Fig. 6.

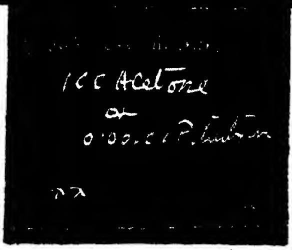

Fig. 8.

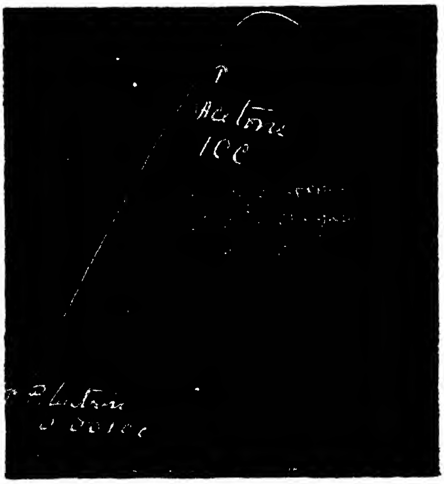

Fig. 7.

Explanation of Plate IV.

Fig. 1—Shows the stimulating effect of antuitrin G (0·1 c.c.) on a guinea-pig's excised non-pregnant uterus in bath.

Fig. 2—Shows the effect of adding pituitrin (0·0001 c.c.) alone and the effect of adding the same dose after the addition of insulin (2 units) to the bath containing an excised guinea-pig's pregnant uterus.

Fig. 3*a*—Shows the stimulating effect of the soluble extract of corpus luteum (P. D. & Co.) (0·1 c.c.) on the excised pregnant uterus of a guinea-pig.

Fig. 3*b*—Shows a similar effect of the soluble extract on a non-pregnant guinea-pig's uterus in bath.

Fig. 4*a*—Shows the inhibitory effect of the watery extract of corpus luteum (Bengal Immunity Co.) prepared for oral administration, on the pituitrin contraction of the cat's excised uterus in bath.

Fig. 4*b*—Shows a similar effect of the watery extract on the rhythmical contractions of a cat's excised uterus in bath.

Fig. 5—Shows the inhibitory effect of thymol in glycerine on the pituitrin contraction of an excised guinea-pig's uterus in bath.

Fig. 6—Shows the absence of reaction to pituitrin (0·0001 c.c. to 0·001 c.c.) when added after thymol in glycerine (0·5 c.c.) to a bath containing an excised guinea-pig's uterus which was very sensitive even to a dose of 0·0001 c.c. of pituitrin. There was a reaction when a stronger dose (0·01 c.c.) was added.

Fig. 7—Shows the inhibitory effect of acetone (1 c.c.) on the pituitrin contraction of the guinea-pig's non-pregnant uterus in bath.

Fig. 8—Shows the absence of reaction when pituitrin (0·001 c.c.) was added along with acetone (1 c.c.) to the bath containing an excised guinea-pig's uterus.

thymol in glycerine (1 c.c.) into a chloralosed non-pregnant cat caused inhibition of the rhythmical contractions. Thymol inhibited also the movements of an excised segment of guinea-pig's small intestine in bath. These results show that thymol has an inhibitory effect on smooth muscle in general. This action of thymol does not appear to have been mentioned anywhere in the literature. As thymol is used therapeutically as an anthelmintic and intestinal antiseptic, its influence on smooth muscle in general and on the pregnant uterus in particular should be taken note of. Moreover, its therapeutic value in non-toxic doses in cases of threatened abortion appears to be worthy of investigation.

Glycerine by itself had a negligible effect on the uterine movements. It caused sometimes a slight relaxation *in vitro* and sometimes had no effect.

Acetone, ether, and alcohol, all had inhibitory effects on the uterine movements *in vitro*. Of these, acetone is of importance as it or its precursor is likely to accumulate in the body or locally in the uterus in conditions of abnormal metabolism or changes caused by hormonic or other agents. Tested *in vitro*, 1 c.c. of acetone caused profound inhibition of the uterine contraction brought about by 0·001 c.c. of pituitrin as in Plate IV, fig. 7. The same dose of pituitrin, when added along with acetone or after the addition of acetone, produced no reaction as in Plate IV, fig. 8. When stronger doses were added there was a feeble contraction. After the action of the chemical agents mentioned above, the uterus in bath rapidly recovered its tone on washing and reacted normally again to pituitrin, showing thereby that they had no damaging effect on uterine muscle.

Bile acids, which were at one time credited with inhibitory influence on the uterine movements, were also tried *in vitro* in a few of the experiments. Sodium taurocholate, 0·1 to 1 c.c. of 1 per cent solution, had sometimes a slight inhibitory effect after a minute's delay and sometimes had no effect.

SUMMARY.

1. Experiments were performed on guinea-pigs, cats, and rats, pregnant and non-pregnant, with a view to find out the possible agents in the body other than the corpus luteum hormone which have an inhibitory influence on uterine motility.

2. The effects of antuitrin S, antuitrin, antuitrin G (P. D. & Co.), thyroxine, adrenaline, insulin and vitamin E on uterine motility were studied *in vivo* and *in vitro*, after a series of injections into normal and ovariectomized guinea-pigs, and normal cats and rats, pregnant and non-pregnant.

3. The results were negative except in the case of adrenaline which had an inhibitory effect on the pregnant as well as on the non-pregnant uterus of rat and guinea-pig. Antuitrin and antuitrin G (P. D. & Co.) were found to be excitatory. Insulin intensified the pituitrin reaction of the pregnant and the non-pregnant uterus of guinea-pig. Vitamin E had no direct influence on the uterine motility but promoted the growth of luteal tissue.

4. Experimenting with certain chemical substances, it was found that thymol, acetone, ether and alcohol, all had inhibitory effects on uterine motility. Of these,

thymol and acetone had the most profound influence. Bile acids were found to have a negligible effect.

ACKNOWLEDGMENTS.

I wish to express my thanks to Messrs. C. Vareed and P. A. Paul for technical assistance in carrying out the experiments.

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EXTRA-SYSTOLES.

A STUDY OF FORTY-ONE CASES.

BY

T. K. RAMAN, M.D., D.T.M. (Cal.).

(From the Department of Medicine, Medical College and King George Hospital, Vizagapatam.)

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EXTRA-SYSTOLES are otherwise called premature contractions or ectopic beats. The abnormal focus from which the impulse starts might be in the auricle, auriculo-ventricular node, auriculo-ventricular junctional tissue or in the ventricle. If the abnormal focus is in the auricle the condition is called auricular extra-systoles; if in the auriculo-ventricular node or in the auriculo-ventricular junctional tissue, nodal extra-systoles and if in the ventricle, ventricular extra-systoles. In the ventricular form the abnormal focus might arise in the right or in the left ventricle. (New nomenclature is used throughout.)

There were 41 cases in this series * and cases diagnosed by electrocardiogram only are included.

Age.—The minimum age was 14 and the maximum 60 years.

Sex.—There were 4 females and 37 males.

Ætiology :

(1) *Myocarditis of unknown origin.*—Fifteen cases (33 per cent) belong to this group. Wassermann reaction was negative in these cases.

(2) *Rheumatic infection* was manifested in 11 cases (25 per cent). Nine cases showed evidence of double mitral lesion and one case showed mitral and aortic lesion.

(3) *Syphilis* was manifested as aortic regurgitation in one case and myocarditis with positive Wassermann reaction in three cases.

(4) *Essential hypertension.*—There were only two cases.

* This series consists of 515 patients and 852 electrocardiograms.

(5) *Miscellaneous.*—There were two cases of pulmonary tuberculosis, two of ankylostomiasis, one of beri-beri, one of thyrotoxicosis, and one of coarctation of the aorta. Two cases showed no other abnormality except the extra-systoles.

As far as the first four groups are concerned, viz., rheumatic infection, syphilitic myocarditis, myocarditis of unknown origin and essential hypertension, one can safely say that the extra-systoles are manifestations of those diseases. Regarding the others it is quite possible that the extra-systoles might be co-existent conditions without any relation to the associated disease.

Auricular extra-systoles.—Eleven patients showed auricular extra-systoles in 18 plates. Auricular premature contraction alone occurred in four patients, and in all the other cases it was associated with ventricular extra-systoles. One patient with auricular fibrillation showed auricular extra-systoles when normal rhythm was restored.

Nodal extra-systoles.—Four patients showed nodal extra-systoles in 4 plates, one showed nodal extra-systoles alone without any other abnormality and the other three showed ventricular extra-systoles in addition.

Ventricular extra-systoles.—Eighteen patients showed left ventricular extra-systoles (VPCL) alone, eleven patients showed right ventricular extra-systoles (VPCR) alone, six cases showed both right and left ventricular extra-systoles and one patient showed interpolated extra-systoles with right ventricular extra-systoles. Five cases showed auricular fibrillation in addition to the extra-systoles.

CLINICAL HISTORY OF TEN CASES.

Case No. 1. Hindu male, aged 45 years, a case of essential hypertension with extra-systoles, was admitted on 21st May, 1931, with signs of congestive heart failure, hypertrophied heart, gallop rhythm, a mitrai systolic murmur and a blood pressure of 210 systolic and 110 diastolic. Blood urea was 33 mg. and kidney function was normal. During his stay in hospital 12 electrocardiograms were taken and all of them showed extra-systoles either auricular, ventricular or both. The general condition improved but the extra-systoles persisted at the time of discharge on 30th July, 1931.

Case No. 2. Hindu male, aged 14 years, a case of double mitral lesion with congestive heart failure and extra-systoles, was admitted on 20th June, 1931. He was kept in the hospital for nearly 3 months. The condition of the patient did not improve and he died on 17th September, 1931.

Case No. 3. Hindu male, aged 23 years, a case of myocarditis and heart failure, was admitted on 23rd June, 1931. He showed extra-systoles every alternate beat (pulsus bigeminus) after digitalis. He was discharged on 5th July, 1931.

Case No. 4. Hindu male, aged 35 years, a case of rheumatic endocarditis and mitral stenosis, was admitted on 16th May, 1931. He was kept in hospital for nearly a month and was discharged. He was readmitted after one month with the same physical signs but the general condition was worse and was discharged after a fortnight with persistent extra-systoles.

Case No. 5. Hindu male, aged 40 years, a case of chronic syphilitic myocarditis, was admitted on 4th September, 1931, with signs of heart failure. During his stay in the hospital, his heart was distinctly irregular and showed multiple extra-systoles, auricular and ventricular. He showed in addition ventricular tachycardia. He was discharged on 18th September, 1931. Four days later after discharge, the patient was brought to the out-patient department of the hospital at 2-30 p.m. and died a few minutes later.

Case No. 6. Hindu male, aged 19 years, was admitted on 7th June, 1930, with congestive heart failure, extra-systoles and auricular fibrillation. The patient died and the post mortem showed sclerosing endocarditis of mitral valve.

Case No. 7. Hindu male, aged 15 years, a case of mitral stenosis with congestive heart failure and extra-systoles, was admitted on 31st August, 1933. His general condition was bad, no

improvement resulted by treatment and he died on 8th September, 1933. Post mortem showed pericarditis, dilated heart and mitral stenosis.

Case No. 8. Hindu male, aged 49 years, was seen in 1929 with signs of coronary thrombosis ? and left ventricular failure. He improved after a fortnight and was able to do his work after 3 months. Examination on 18th July, 1934, revealed gross myocardial damage, congestive heart failure and irregular extra-systoles. He was admitted into the hospital on 19th September, 1934, with the same symptoms. Electrocardiograms showed irregular ventricular extra-systoles and ventricular tachycardia. He died on 2nd January, 1935.

Case No. 9. Hindu female, aged 22 years, admitted into the gynic ward, showed an irregular heart. Electrocardiograms showed a series of ventricular extra-systoles.

Case No. 10. Hindu male, aged 35 years, a case of myocarditis, amœbic hepatitis and extra-systoles, was admitted on 19th January, 1937. He showed a series of extra-systoles both auricular and ventricular. The patient was discharged on 22nd February, 1937. He died a few days after his discharge from the hospital.

Case No. 2 was under observation for nearly 3 months, showed three extra-systoles per minute, and died 13 months after the onset of symptoms. In another case the history was indefinite and the patient showed 27 extra-systoles per minute and died a month after E. C. G. was taken. In case No. 5 the symptoms were only of 4 months' duration. The patient had 46 extra-systoles per minute and he died 3 weeks after admission. Case No. 6 was under observation for 4 years. He had 20 extra-systoles per minute, had auricular fibrillation in addition and he died 6 years after the onset of the disease. Case No. 7 showed six extra-systoles per minute and died 2 years after the onset of the disease. Case No. 8 had 45 extra systoles per minute, had ventricular tachycardia in addition and died 9 years after the first onset of heart failure. Case No. 10 had 32 extra-systoles per minute and died 3 months after the first attack of dyspnœa. In three other cases pulsus bigeminus was present due to the administration of digitalis. Seven out of the 41 cases (17 per cent) died. Post mortem was available only in two cases. One case showed sclerosing endocarditis of the mitral valve and the other mitral stenosis and pericarditis.

The minimum period of duration from the onset of the symptoms to the death of the patient was 4 months and the maximum 9 years. From these observations one can safely say that as extra-systoles become more frequent the prognosis gets worse. One is not in a position to say whether co-existent auricular fibrillation will worsen the prognosis. Out of five patients showing auricular fibrillation with ventricular extra-systoles only one patient died.

In the out-patient department one can often spot the extra-systoles in patients coming for some other complaint. The extra-systoles in these cases are only 'incidental'. Extra-systoles in themselves have no clinical significance. But when associated with heart failure or other cardiac abnormalities, either clinically or electrocardiographically, they indicate myocardial damage. Parsonnet and Hyman (1929) call this 'fundamental group'. Frequent or persistent auricular extra-systoles might lead to the graver condition of auricular fibrillation (East and Bain, 1936). Extra-systoles occurring every alternate beat (pulsus bigeminus) may be due either to the administration of digitalis or as a result of gross myocardial damage. Excitement, a hot cup of coffee, a cool drink or a smoke might bring out latent extra-systoles.

Explanation of Plate V.

Fig. 1. (Lead II). Auricular extra-systole from a case of aortic regurgitation. P in the extra-systole is erect and P-R interval is reduced to 0·06 second. The pause after the extra-systole is not compensatory.

Fig. 2. (Lead III). Auricular extra-systole from a case of chronic myocarditis. Auricular complex is abnormal and P is inverted.

Fig. 3. (Lead II). Auricular and ventricular extra-systoles from a case of syphilitic myocarditis. There is only one auricular extra-systole (third beat) and the others are ventricular. Usually the ventricular complex in auricular extra-systole is normal. This figure shows the rare form of ventricular complex.

Fig. 4. (Lead II). Auricular and ventricular extra-systoles from a case of chronic myocarditis (case No. 10). There are three extra-systoles, one auricular and two ventricular.

Fig. 5. (Lead II). From the same case as above. The two ventricular extra-systoles are seen. The auricular extra-systole seen in Fig. 4 is blocked and is not seen.

Fig. 6. (Lead I). Nodal extra-systole from a case of rheumatic heart. The third beat is the extra-systole, and P is seen after the R.

Fig. 7. (Lead II). Ventricular extra-systoles from a case of rheumatic endocarditis and mitral stenosis. Second beat is a right ventricular extra-systole and the fourth, left ventricular extra-systole.

PLATE V.

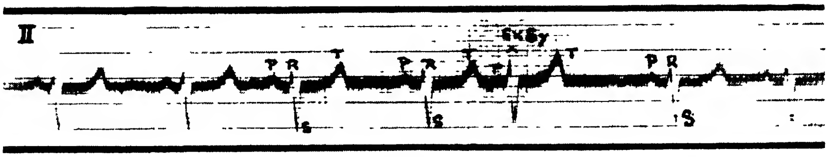


Fig. 1.

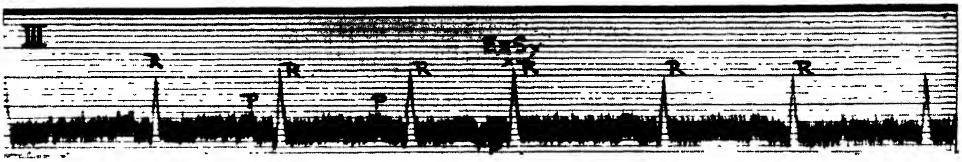


Fig. 2.

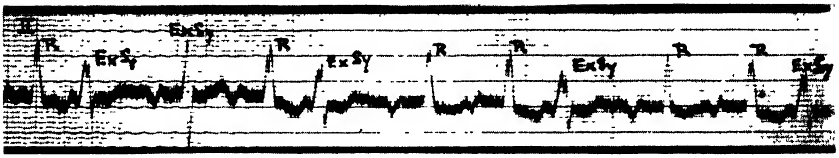


Fig. 3.

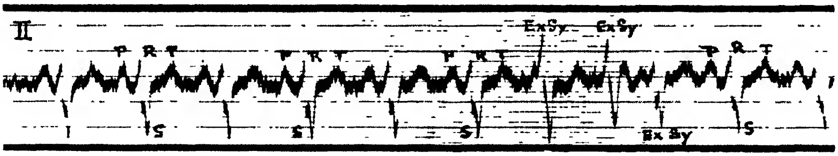


Fig. 4.

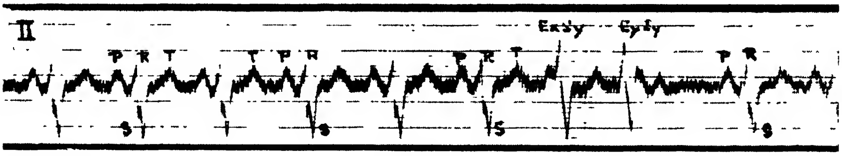


Fig. 5.

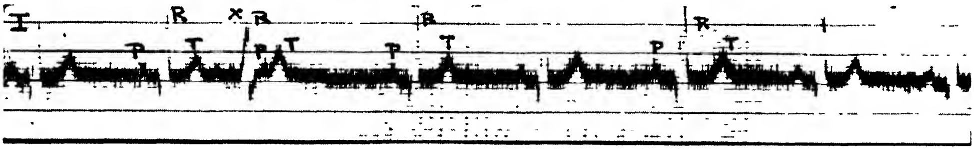


Fig. 6.

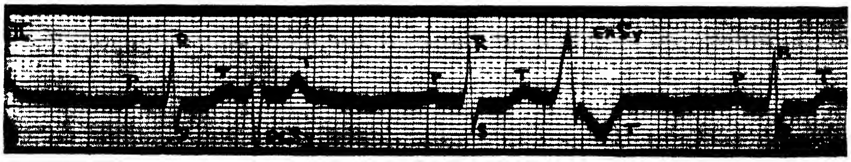


Plate VI.

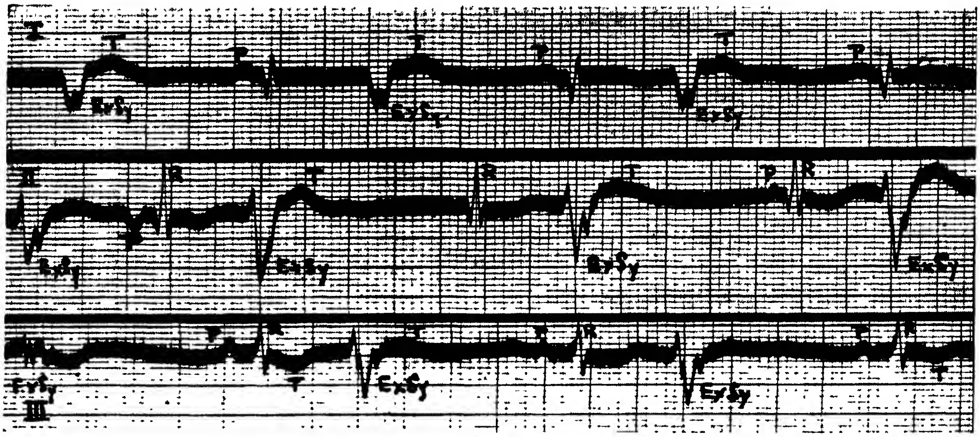


Fig. 8.

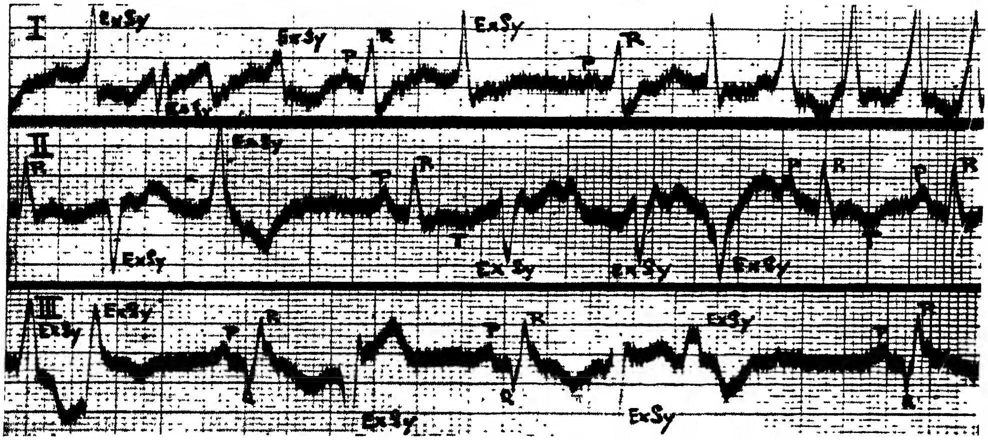


Fig. 9.

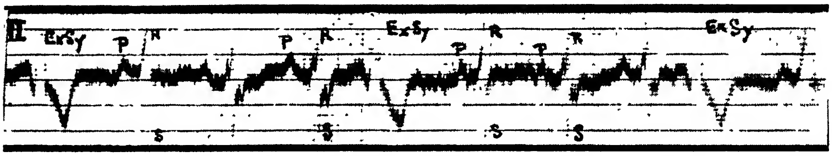
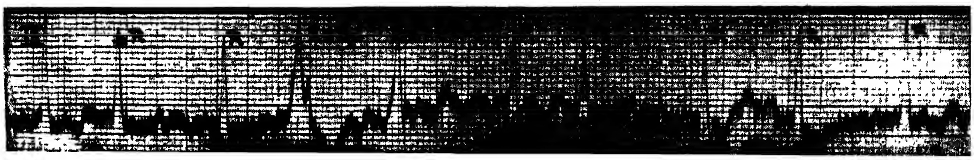


Fig. 10.

Fig. 11.



EXPLANATION OF PLATE VI.

Fig. 8. (Leads I, II and III). Ventricular extra-systoles both right and left (pulsus bigeminus) from a case of chronic myocarditis and heart failure. In lead I all the extra-systoles are left ventricular; in lead II all are right ventricular; in lead III the first beat is a left ventricular extra-systole, the other two extra-systoles are from the right side.

Fig. 9. (Leads I, II and III). Multiple extra-systoles with ventricular tachycardia from a case of chronic syphilitic myocarditis. Lead I shows only two normal beats, and the last five beats represent a paroxysm of ventricular tachycardia. Lead II shows only four normal beats and lead III only three and the rest are ventricular extra-systoles. This patient in another E. C. G. showed five paroxysms of ventricular tachycardia.

Fig. 10. (Lead II). Left ventricular extra-systoles occurring every fourth beat from a case of essential hypertension and heart failure (case 1).

Fig. 11. (Lead III). Right ventricular extra-systoles from a case of rheumatic heart. The third beat is an interpolated extra-systole, and the seventh beat is an extra-systole with a compensatory pause.

Fig. 12. (Lead III). Left ventricular extra-systoles with auricular fibrillation from a case of syphilitic myocarditis.

Diagnosis of extra-systoles.—The diagnosis of extra-systoles can always be made at the bedside by: (1) Irregular pulse: an occasional extra-systole can be detected only if the patient is examined for a sufficiently long time, and sometimes it might even then be missed. Extra-systoles may occur every alternate beat (pulsus bigeminus) or every third beat (pulsus trigeminus), or once in every fourth or fifth beat. When the pulse is absolutely irregular the condition cannot be diagnosed clinically without the help of the electrocardiogram. (2) The irregularity disappears on exertion but in some cases it still persists. (3) Auscultation: in auricular extra-systoles the extra beat will simulate the normal beat, while in the ventricular form, it will be different from the normal two sounds. (4) On looking at the jugular vein an abnormal (a) wave is seen in a case of auricular extra-systoles.

Electrocardiographic diagnosis.

1. Auricular extra-systoles: P might be normal (Plate V, fig. 1) or inverted (Plate V, fig. 2). The nearer the abnormal focus to the sino-auricular node, the more will it simulate the normal contraction. P-R interval is usually reduced. Ventricular complex is usually normal but occasionally abnormal ventricular complex can be seen (Plate V, fig. 3). The pause after the extra-systoles is not compensatory. In rare cases the pause may be compensatory. Occasionally the premature contraction may be blocked.

2. Nodal extra-systoles (Plate V, fig. 6): P might or might not be seen. If seen it is found after the R. Ventricular complex is normal. The pause is compensatory.

3. Ventricular extra-systoles:—

(*a*) Left ventricular extra-systoles: The ventricular complex is always abnormal and the curve is biphasic. The initial deflection is down in lead I and up in lead II and lead III. In old terminology the initial deflection is up in lead I and down in lead II and lead III.

(*b*) Right ventricular extra-systoles: The initial deflection is up in lead I and down in lead II and lead III. In old terminology the initial deflection is down in lead I and up in lead II and lead III. It is sometimes difficult to find out whether the extra-systoles arise from the left or right ventricle. The abnormal complexes in lead II and lead III are much more significant as to the origin of the extra-systole than in lead I. Practically there is no difference in the clinical significance of extra-systole whether it is from the right or from the left ventricle. P is merged in the ventricular complex and occasionally it might be seen either before or after the ventricular complex. Ventricular extra-systoles might occur as an occasional phenomenon, or as a series of left and right alternating. The pause after the extra-systoles is always compensatory. In the irregular ventricular extra-systoles the normal contour of the extra-systoles will not be seen.

4. Interpolated extra-systoles (Plate VI, fig. 11): The extra-systole occurs between two normal beats and the contour of the extra-systoles will be the same as in the other ventricular extra-systoles, the initial deflection will show from which ventricle it occurs. The sinus rhythm is not disturbed.

SUMMARY.

1. Forty-one cases of extra-systoles were investigated.

2. Four cases were syphilitic, nine cases were rheumatic, and fifteen cases were due to myocarditis of unknown origin.

3. Extra-systoles in themselves have no clinical significance. The prognosis gets worse as the extra-systoles become frequent and persistent.

ACKNOWLEDGMENTS.

My thanks are due to Dr. G. Dinker Rau and Dr. P. Kutumbiah for the clinical aspect; to Dr. P. Ramachandra Rao for the post-mortem notes of cases Nos. 6 and 7, and to Dr. P. Kesavaswamy for the prints of the electrocardiograms.

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Ind. Jour. Med. Res., **28**, 1, July, 1940.

ADDENDUM

To paper entitled ' On Wassermann Reaction. Part V. The Complement ' by S. D. S. Greval *et al.*, *Ind. Jour. Med. Res.*, **28**, 1, p. 257.

The writers' scheme for the titration of the complement is slightly different from that of method No. IV of the Medical Research Council, although it accomplishes the same purpose. The dilutions are made from a 1 in 10 dilution. They are : 1 in 20, 1 in 30, 1 in 40, 1 in 50, 1 in 60, 1 in 70, 1 in 80 and 1 in 90. Only two rows of tubes, one behind the other, are used. One volume of a dilution is put into the front tube which has no antigen and two volumes (of the same dilution) into the hind tube which has antigen.

The difference though apparent from the procedure has not been clearly stated in the communication.—*S. D. S. G.*, 4th July, 1940.

Ind. Jour. Med. Res., **28**, 1, July, 1940.

ON WASSERMANN REACTION.

Part V.*

THE COMPLEMENT.

BY

LIEUT.-COLONEL S. D. S. GREVAL, I.M.S.,
Imperial Serologist, Government of India,
CAPTAIN S. N. CHANDRA (late I.M.S.),
First Assistant,

AND

B. C. DAS, B.SC.,
Chemist.

(From the Imperial Serologist's Laboratory, School of Tropical Medicine, Calcutta.)

[Received for publication, March 31, 1940.]

IN the Wassermann reaction comparatively very little attention has been paid to the quality of the complement. That complement titre of which is below a certain figure should not be used, that there should not be a difference of more than one tube between its titre with and without the antigen, and that its hæmolytic power (without the antigen) and its capacity for being fixed with an antigen-antibody system do not necessarily run parallel, are known. This knowledge is neither very helpful nor exact and as often as not leads to compromises. Certain fractions of the complement ending with the 'fourth' complement have also been known for many years. They have no utility in the Wassermann reaction. Studies

* For Part IV see *Indian Journal of Medical Research*, Vol. **27**, No. 2, 1939, p. 589.

in the quantitative determination of the fixation of complement by immune serum and antigen have been undertaken (Wadsworth *et al.*, 1931 ; Maltaner and Maltaner, 1935) and equations approaching those in colloidal chemistry worked out. No such equations can possibly hold in the Wassermann reaction because the quantity fixed undoubtedly varies with the quality.

In this communication will be dealt with (*i*) a new method of comparing different samples of complement with regard to optimal reaction and titre, (*ii*) complement of optimal reaction but not of optimal titre and the necessary adjustment, (*iii*) cholesterol-shy and cholesterol-proof complement and the necessary adjustments, and (*iv*) reading of results in test proper by the help of the titrated controls when the adjustments have not been altogether successful. General observations will also be made on (*v*) the writers' findings on complement, including some which run counter to current beliefs. Lastly, after drawing attention to an important difference between the original technique and the modified techniques, (*vi*) a suggestion will be advanced that the Wassermann reaction should be re-named Lecithin Complement Fixation (L.C.F.).

I. A NEW METHOD OF COMPARING DIFFERENT SAMPLES OF COMPLEMENT WITH REGARD TO OPTIMAL REACTION AND TITRE.

The titrated controls from 'Strongly Positive' (+++, fixing complement with uncholesterinized alcoholic heart extract antigen) pooled sera have been described in this *Journal* previously (Greval, Das and Sen Gupta, 1938). Two dilutions of the pooled serum are put up in four tubes. They give the following reactions :—

SET 1. With 3 m.h.d. of complement and cholesterinized antigen.		SET 2. With 5 m.h.d. of complement and cholesterinized antigen.	
1st dilution (tube 1).	2nd dilution (tube 2).	1st dilution (tube 3).	2nd dilution (tube 4).
+	T or ±	T or ±	± or –

The dilutions may be 1 in 100 and 1 in 200, 1 in 50 and 1 in 100, or 1 in 25 and 1 in 50. When a large number of sera, 25 or more, have been pooled the dilutions usually are 1 in 50 and 1 in 100. The second dilution always has half the strength of the first.

The antigen is standardized as has also been described in this *Journal* (Greval, Chandra and Das, 1939).

These reactions in the controls are obtained when the complement satisfies the requirements of method No. IV of the (British) Medical Research Committee (now Council, 1918) regarding the correspondence between the front row and the hind row of tubes (with and without the antigen) in its titration. The complement then is of such a quality that neither more nor less than 1 m.h.d. of it is rendered inert by the cholesterinized antigen. This is the Optimal Reaction. Further, the reactions are typical and easily differentiated from one another when the m.h.d. of a complement of optimal reaction is 1 in 40, 1 in 50, or 1 in 60. These m.h.d. denote the Optimal Titre.

When the complement does not give the optimal reaction or when the titre is below or above the optimal titre the reading of the controls is altered. The differentiation between them disappears. Either the second dilution becomes like the first (more positive) or the first dilution becomes like the second (more negative).

The antigen and the serum both being constant the deviations from the expected reactions are entirely due to the peculiarities in the complement as manifested by the lack of the optimal reaction or the titre which is other than optimal.

Incidentally, it may be remarked that the weaker dilution of the serum (tube 2) with the weaker dilution of the complement (set 1) shows appreciably less inhibition of lysis (i.e., less fixation) than the stronger dilution of the serum (tube 3) with the stronger dilution of the complement (set 2), although the relative quantities of the serum and the complement are the same (for instance, 1/100 serum dilution + 3 m.h.d. complement, functionally equalling 2 m.h.d., and 1/50 serum dilution + 5 m.h.d. complement, functionally equalling 4 m.h.d., are arithmetically identical). The antigen is in excess in both cases. In other words, halving the serum gives better differentiation than doubling the complement. This is an argument in favour of keeping the complement constant and varying the serum in those complement-fixation reactions in which doubtful reactions are to be eliminated.

II. Complement of optimal reaction but not of optimal titre and the necessary adjustment.

The complement of optimal reaction and optimal titre gives both the expected reactions and the expected differentiation with the titrated controls. As the titre (reaction remaining optimal) rises above the optimal, to 1 in 70, 1 in 80 and 1 in 90, the fixation increases. The differentiation between the two controls is lost, first in the 1st set and later in the 2nd. As the titre falls below the optimal, to 1 in 30, 1 in 20, the fixation decreases. Traces of lysis appear in the 1st tube of the 1st set and the 2nd tube shows almost complete or complete lysis. The 2nd set also shows corresponding changes.

The adjustment consists of being liberal in fixing the dose when the titre is high and stringent when the titre is low.

The liberality is exercised by (*i*) insisting on the tube giving the titre being perfectly *crystal clear* and then, in addition, by (*ii*) taking as the working dose one tube below. For instance, the reaction may be :—

	Dilutions of complement.			
	1 in 60	1 in 70	1 in 80	1 in 90
Hind row (2 volumes of dilution, with antigen).	—	—	(?)—	±
Front row (1 volume of dilution, no antigen).	—	—	(?)—	±

(?)— indicates a smaller degree of inhibition of lysis than ?—. The tube is fully lysed for all practical purposes and would be accepted as such if a clearer tube did not exist.

The observed m.h.d. is 1 in 70. The working dose will be 1 in 60.

Titration beyond 1 in 90 is not carried out. When the 1 in 90 is the observed dose, 1 in 80 is the working dose. On a few occasions when the observed dose has been 1 in 100 satisfactory reactions have been obtained with a working dose of 1 in 80. The excess of the complement of this type used is balanced by the increase in fixation which results.

The stringency is exercised by accepting as fully lysed a tube which is not crystal clear. For instance the reaction may be :—

	Dilutions of complement.		
	1 in 20	1 in 30	1 in 40
Hind row (2 volumes of dilution, with antigen).	—	(?)—	±
Front row (1 volume of dilution, no antigen).	—	(?)—	±

The observed dose is 1 in 20. The working dose will be 1 in 30. Wyler (1929) takes his titre from such a tube always, regardless of the titre.

Some time ago a low titre of the complement used to be a frequent source of annoyance in this laboratory. With better conditions of feeding, housing and bleeding the guinea-pigs it has disappeared. Now a titre below 1 in 20 is never and 1 in 20 hardly ever found. The complement of the latter titre can be used and results read by comparison with the reactions of the titrated controls (*vide infra*).

III. Cholesterol-shy and cholesterol-proof complement and the necessary adjustments.

The reaction of the complement with the cholesterinized antigen, in its titration (regardless of the titre), brings out two more types, in addition to the complement of the optimal reaction. They are: (1) cholesterol-shy complement and (2) cholesterol-proof complement.

(1) *Cholesterol-shy complement.*

Unlike what happens in the complement of the optimal reaction, more than 1 m.h.d. of this complement (read from the tubes without the antigen) is rendered inert by the cholesterinized antigen. The degree and the manner of this occurrence differ, thus :—

(*i*) The fully lysed tube in the hind row (with the antigen) may lag one or more places behind the fully lysed tube in the front row (without the antigen).

(*ii*) The lysis in the hind row may follow the lysis in the front row fairly well but may not be really complete in any tube (?—).

(*iii*) The correspondence in the hind row may be confined to the middle zone only, inhibition of lysis occurring both in the beginning and in the end.

(*iv*) The lysis in the hind row may be poor (±).

(*v*) The lysis in the hind row may be poor and irregular (in the middle or towards the end).

(*vi*) There may be no lysis in the hind row or merely a trace (+ or T).

The adjustment consists of either increasing the complement or reducing the cholesterol content by adding more heart extract to the combined antigen thus :—

For (*i*), which occurrence is not frequent, method No. IV of the Medical Research Council provides that the tube in the front which corresponds to the fully lysed tube in the hind row should be taken for the m.h.d. (not the tube ahead of it) when there is a difference of one tube only. When the difference is of more than one tube, rejection of the complement is recommended. In the writers' experience even a difference of more than one tube is immaterial. M.h.d. is taken from the tube in front of the fully lysed tube of the hind row. The resulting excess of the complement of this type is compensated by the increase of fixation which also results.

For (*ii*) and (*iii*) the combined antigen 2 parts is diluted with heart extract 1 part.

For (*iv*) and (*v*) the combined antigen is diluted with an equal volume of heart extract.

For (*vi*) the combined antigen 1 part is diluted with heart extract 2 parts.

In adjusting the difference for (*ii*), (*iii*), (*iv*), (*v*) and (*vi*) the cholesterol content is reduced.

Adjustment for the titre (below or above the optimal) is not made.

Controls are put up with the test proper of the modified antigen with 2, 3, and 5 m.h.d. of the complement.

In the writers' procedure the Wassermann reaction is done with three antigens (Greval, Chandra and Das, *loc. cit.*). With a complement of this type the other two antigens also give reactions which incline unduly towards the positive side. This fact is taken into consideration in reporting the results.

Complement of this type also used to be frequent in this laboratory especially in the cold weather (December to February). It has disappeared with the improvement in the life of the guinea-pigs. That it was due to deficiency or discomfort, not to an infective disease, is concluded by the absence of increase in mortality in the animals during the period.

The defect in the complement responsible for the irregularities (*i*), (*ii*), (*iii*), (*iv*), (*v*) and even (*vi*) tends to disappear on the addition of normal (negative, human) serum. Therefore, if, regardless of the defect, the complement is used in a day's work there will be some negative results in the batches of sera tested. Even the known negative serum may remain negative. The titrated controls, however, will show that the readings are more positive.

The term cholesterol-shy is used in preference to cholesterol-labile because of the partial labileness.

(2) *Cholesterol-proof complement.*

Contrary to what happens in the case of the complement of the last type, less than 1 m.h.d. of the complement of this type is rendered inert by the antigen. The result is that with the usual allowances of 3 m.h.d. and 5 m.h.d. in the test an excess is left. Further, the titrated controls show that this type of complement is not so fixable as the complement of the optimal reaction although it shows a similar increase in fixability with the rising titre.

Incidentally, for the detection of this peculiarity in a complement of high titre an extra tube in the titration is necessary. It is put up with :—

	Volume.
Complement, 1 in 90 ..	.. 1 (instead of 2).
Antigen dilution ..	.. 1
Saline	.. 1
Sensitized r.b.c. ..	.. 1

Otherwise, if the 1 in 90 tube in the front row is fully lysed and the corresponding tube in the hind row is also fully lysed, it is not possible to say whether the complement is of the optimal reaction or of the cholesterol-proof type. If the extra tube is lysed, even partially, in 30 minutes' incubation, the complement

may safely be presumed to be of the latter type. The complement of the former type when going beyond 1 in 90 can produce in the tube only a trace of lysis which cannot be made out until the r.b.c. have settled down.

Adjustment is made either by restricting the dose of the complement or by modifying the antigen.

The restriction of the complement is effected thus: Use $2\frac{1}{2}$ m.h.d. and $4\frac{1}{2}$ m.h.d. instead of 3 m.h.d. and 5 m.h.d., respectively, if the last clear tube in the front row (without antigen) as given in the column ' front row ' is accompanied by the last clear tube in the hind row (with antigen) as given in the column ' hind row ' and if in the case of the last (1 in 90) tube the extra tube is also lysed.

Front row.	Hind row.	
1 in 20 ..	.. 1 in 30	
1 in 30 ..	.. 1 in 50	A ? — reaction can be accepted as — in the hind row of all tubes.
1 in 40 ..	.. 1 in 60	
1 in 50 ..	.. 1 in 80	
1 in 60 ..	.. 1 in 90	
1 in 90 ..	.. 1 in 90	also the extra tube lysed even partially.

For other combinations restriction is not attempted but *a lowering of fixation is anticipated.*

No adjustment is made for the titre higher than optimal.

The modification in antigen is effected by diluting the combined antigen with 0·25 per cent phenolized saline (instead of ordinary saline). The dose of the complement then is not restricted.

The writers in their routine restrict the dose of the complement. The modified antigen is used for chosen cases. *Extra-titrated controls then are put up with the same antigen.*

On the whole the fixation with this type of complement is lowered even when the dose can be restricted. The type represents the commonest irregularity of the complement. While the writers have succeeded in practically abolishing the other irregularities with better care of the animal this irregularity remains unaffected. In fact it has increased in frequency and appears to indicate that it may be the normal reaction of the complement of particularly healthy animals.

So far as the writers are aware no observations have been made on this type of complement.

The term cholesterol-proof is used in preference to cholesterol-stabile because of the fact that the stabileness goes beyond the limit of the normal complement (complement of optimal reaction).

It need hardly be added that the complement discussed is the pooled complement of at least three guinea-pigs.

IV. Reading of results of the test proper when adjustments have not been altogether successful, have failed completely, or have not been made for small irregularities.

Two sets of titrated controls, set 1 and set 2, have been described. One more set, set 3, put up with the same dilutions of the pooled positive serum but with 2 m.h.d. of complement and Bordet's cholesterinized antigen, is also used routinely. When phenolized antigen is used in the test, controls are put up with it also; they are set 1 phenolized and set 2 phenolized. These three (or five) sets of controls, even when they are slightly defective at times, as a rule indicate how the reactions of certain sera in the test proper should be read.

When the fixation inclines unduly towards the positive side, the second tubes of the sets become like the first (more inhibition of lysis, more positive). The results are not unsatisfactory as long as the second set shows the difference plainly. When the difference in the second set is also lost, all ++, +, T and ± readings should be rejected; all ? — readings should be read —; and of course all — readings should be accepted. All +++ readings should also be accepted; a slightly less degree of fixation with more accurately adjusted antigen-complement system may reduce them to ++T which will be reported +++ just the same. A record is kept of the failure of adjustment so that a slight difference between the reactions reported at the time and those reported at a later date may be explained.

When the fixation inclines unduly towards the negative side, the first tubes of the sets become like the second (more lysis, more negative). The results are not unsatisfactory as long as the first set shows the difference sharply. When the sharp difference in the first set is also lost and a T appears in the place of + all —, ? —, ± readings should be rejected; all T (not TT) readings should be read + as long as the colour does not exceed that of the 1st tube of the 1st set; and of course all +, ++ and +++ readings should be accepted.

The reaction with Bordet's antigen will also help in deciding whether a mere trace of lysis is significant or not. It must, however, be remembered that a cholesterol-shy complement for which no adjustment is made in the Bordet's antigen-complement system gives a higher reading with this antigen.

From what has been said, concerning the conditions which must be fulfilled in the controls before the reading of the reactions of a serum under investigation can be accepted, it might appear that the rejection of a day's work is a common occurrence. Such is not the case. The adjustments are nearly always satisfactory. The important conclusion, however, emerges that without satisfactory adjustments in the antigen-complement system certain reactions not possessing enough latitude, such as the reactions of sera from borderline cases, are always likely to be untrustworthy and unrepeatable.

V. Writers' other findings on the complement including some which run counter to common beliefs and associated considerations.

(1) *Further observations on cholesterol-shy and low-titre complement.*

These troubles are very common in Calcutta and are responsible for devices which get over the requirements of the methods advocated by the (British) Medical Research Council. Excess of untitrated or titrated complement is frequently used. Antigens which are not anti-complementary are used regardless of their worth.

The cholesterol-shy complement is mostly encountered in the cold weather (November, December and January) and has been controlled by the writers by giving the guinea-pigs Marmite (2 teaspoonfuls to 100 animals, daily). The low-titre complement occurs in the hot weather (dry and wet, the remaining months of the year) and has been controlled sufficiently by leaving ice in the animal house from 11 a.m. until sunset, in addition to giving Marmite.

The Marmite in diet has on the whole resulted in a yield of cholesterol-proof complement.

Artificially, for experimental purposes, cholesterol-shy complement can be obtained from a complement which is normal by keeping the latter indifferently frozen for several weeks. Low-titre complement can also be obtained from a normal complement by preserving the latter for long periods (months) by means other than freezing hard.

Cholesterol-shy and low-titre complement are not linked.

(2) *Preservation of complement.*

It is known that complement frozen hard keeps indefinitely.

Dilutions of complement (in saline) keep their titre for all practical purposes constant for a week or so in a refrigerator (temperature 2 to 8°C.). The slight loss is not measurable by the scheme of titration usually employed.

Complement salted (partly following Friedberger, quoted by Browning, 1931) with sodium chloride (0·34 g.* to 10 c.c.) and kept in a refrigerator keeps its titre for weeks. The loss occurs very slowly.

Complement dried rapidly (on Whatman filter-paper, such as is used in blood work, or free at the bottom of small phials) *in vacuo* and in the cold also keeps well in a refrigerator for weeks. When salted, treated and kept as above, it keeps for months.

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* Normal saline is 0·85 per cent sodium chloride. For a 1 in 5 dilution of 100 c.c. of complement 4 volumes of saline will be added. The added fluid may be distilled water if 0·85 g. × 4 = 3·4 g. of sodium chloride has been added in advance to the complement. For 10 c.c. of complement the quantity will be 0·34 g.

For preparing dilutions from salted complement, wet or dry, first distilled water is added to reduce the sodium chloride content to 0·85 per cent, then the required dilution is made with normal saline.

For preparing dilutions from unsalted dry complement the loss of water is first restored by distilled water then the dilution is made with normal saline.

To all stored complement, other than the one frozen hard, an accidental failure of a refrigerator which is well insulated against heat, once in a way, does not do any appreciable harm. The failure may last many hours, as occurs in a laboratory closing at 4 p.m. and opening at 10 a.m. next day.

(3) *Serum-protein content of complement dilution.*

In the usual scheme of titration of complement the serum-protein content of the complement dilutions decreases steadily. It can be kept constant by making dilutions from an initial 1 in 10 dilution of an active complement with another 1 in 10 dilution of an inactivated complement. The m.h.d. (titre) is then found to be higher.

The same effect is produced by adding serum proteins from the sera in the test proper. Two, 3, and 5 m.h.d. of the complement added in the test, thus, yield more than the quantity indicated. Contrary to the common belief a normal serum is not anti-complementary but pro-complementary. Further, the normal serum also protects the complement from the anti-complementary action of the antigen. The allowance of 1 m.h.d. made for the antigen, thus, leaves some complement free.

(4) *Anti-complementary sera.*

The syphilitic sera, sera in which micro-organisms have grown and sera from cases of certain diseases have a definite anti-complementary effect. The 'inactivation' of the sera before the test is not done so much to remove the native complement as to remove the anti-complementary effect due to the growth of the micro-organisms. Amongst the diseases which make the serum anti-complementary kala-azar ranks first (Greval, Sen Gupta and Napier, 1939); dilutions of 1 in 25 (instead of the usual 1 in 5) are occasionally found to inhibit lysis completely in the serum control.

In this laboratory the anti-complementary effect encountered in the test, apart from that of kala-azar cases, is very little. This is due to the fact that a great majority of the sera tested are fresh. The test is done on five days of the week. When a serum has to be kept for a day or so it is prepared for the test by removal of the clot (if any), clarification by centrifuging and inactivation, and kept (not frozen) in a refrigerator. It is not re-inactivated before use.

(5) *Magnitude and constancy of the m.h.d. of complement.*

The smaller the quantity of the complement in an m.h.d. the more sensitive is the test. The quantity is determined by the concentration of the sensitized

r.b.c. suspension. Other things being equal, a technique using 5 per cent red cell suspension would be less sensitive than the one using 3 per cent. A constant concentration of the red cell (whatever the percentage may be) is necessary for the constancy of the m.h.d. of the complement. The red cell suspension, therefore, must be standardized.

In Wyler's technique (*loc. cit.*) of the Wassermann reaction the method for standardizing the red blood cell suspension yields a suspension which is a little over 3 per cent. Besides, the laboratory must be supplied with coal gas.

The senior writer (Greval, 1929; Greval, Yesudian and Choudhury, 1930) also described a method for standardizing the red cell suspension for the requirements of the method No. IV. The suspension obtained is 3 per cent, supply of coal gas is not necessary and calculation does not go beyond simple arithmetic. He has used this method now for over ten years and finds it satisfactory.

(6) *Delayed lysis in the cold.*

The writers leave their racks of tubes in a refrigerator overnight with a view to detecting traces of lysis. Kolmer (1929) thinks that this practice leads to a delayed lysis and a small percentage of falsely negative results. In the writers' opinion such a lysis, when it occurs, is not delayed for more than half an hour or so and occurs while the red blood cells are still suspended, otherwise the tube would show a colourless column of fluid over a coloured column. Further, it is always associated with two conditions *acting together:* (*i*) paucity of the reagin in the serum and (*ii*) cholesterol-proof complement or an over-adjustment for cholesterol-shy complement. The titrated controls prove all these statements: (*i*) the trace of lysis in the positive control, when it occurs, is uniform, not resting over deposited red cells, under a colourless column of fluid; (*ii*) the reagin in the titrated positive controls is minimum for a positive reaction and (*iii*) the trace of lysis occurring contrary to expectation is associated with either a cholesterol-proof complement not fully adjusted or an over-adjustment for a cholesterol-shy complement. The delayed lysis, therefore, is not likely to lead to a false report. The result is read correctly by the help of the titrated controls.

(7) *Two doses of the complement versus two dilutions of the serum.*

It has already been stated that an increase in the complement brings out the differences in fixation less than a decrease in serum. Further, decreasing the serum is more economical than increasing the complement. Furthermore, decreasing the serum guards against a low positive (T) or doubtful reaction resulting from a paradoxical effect of a high concentration of the reagin in the serum. Such an effect has been observed in complement-fixation in kala-azar (Greval, Sen Gupta and Napier, *loc. cit.*), in which increasing dilutions of certain sera have given increasing degrees of fixation.

A paradoxical Wassermann reaction, however, does not appear to occur when the technique of the British methods or of methods planned after the British

methods is followed. In the writers' technique which is planned after method No. IV of the Medical Research Council, three antigens are employed. One of the antigens (uncholesterinized alcoholic heart extract antigen) reacts only with the sera in which the reagin is likely to exist in a high concentration, such as in cases of secondary syphilis (strongly positive reaction, +++, of the writers or even ++T and ++±). None of these cases have been found to give less than a complete inhibition (++) with the two doses of the complement (3 m.h.d. and 5 m.h.d.) and the ordinary antigen (cholesterinized alcoholic heart-extract antigen). A reaction falling short of full inhibition will be looked upon as anomalous and repeated with two dilutions of the serum, 1 in 5 (the usual dilution) and 1 in 10. Lest there should be some sera in which the reagin though not of the quality which reacts with the uncholesterinized antigen is yet in a high enough concentration to yield a paradoxically low positive reaction (TT), all low positive (TT) reactions are repeated with two dilutions of the serum.

Kolmer (*loc. cit.*) recommends the serum dilution method in complement-fixation from the standpoint of economy, accuracy and ease of manipulation.

Basu and Chatterjee (1936) also find variation in serum more satisfactory than variation in complement. They, however, appear to have derived their conclusion mostly from an increase in the concentration of the serum dilution. Their dilutions are 1 in 2, 1 in 4, and 1 in 8 instead of the usual 1 in 5 dilution of a standard method they still follow.

The writers from their experience of the reactions of the titrated controls conclude that decreasing the serum to a volume of a 1 in 10 dilution instead of increasing the complement (to 5 m.h.d., the 2nd dose of the method) will convert a certain number of doubtful reactions into negative.

(8) *System of recording fixation.*

Some workers, following method No. IV (Medical Research Council), record the m.h.d. of complement fixed thus :—

(*i*) +++, serum fixes (= at least fixes) 8 m.h.d. of complement (Iyengar, 1919),

(*ii*) ++, serum fixes (= at least fixes) 5 m.h.d. of complement, or

(*iii*) +, serum fixes (= at least fixes) 3 m.h.d. of complement.

The present writers do not put up the test with 8 m.h.d. of complement. But when they reduce the dose from 5 m.h.d. and 3 m.h.d. to 4½ m.h.d. and 2½ m.h.d., respectively, in the adjustment of the antigen-complement system, they still assign to the fixation the original values of 5 m.h.d. and 3 m.h.d.

They call a serum :—

(*i*) +++, strongly positive, when it fixes with uncholesterinized antigen 4 m.h.d. of complement (= 5 m.h.d. with cholesterinized antigen which is anti-complementary to the extent of 1 m.h.d.).

(*ii*) ++, positive, when it fixes 3 m.h.d. (fully, +) and 5 m.h.d. (+ or T) of complement with cholesterinized antigen.

(*iii*) +, weakly positive, when it fixes only 3 m.h.d. (fully, +) of complement with cholesterinized antigen.

(9) *Interdependence of titrations, standardizations and adjustments. Importance of complement.*

The constancy of the titrated positive controls, the standardization of the antigen and the adjustment of the antigen-complement system (including reduction in the cholesterol content) are interdependent. Easiest of the standard reagents to come by is the complement of optimal reaction. Although the necessary adjustments make it possible to work with other types of complement also, the writers titrate their pooled positive sera on a day when they come by a complement of optimal reactions. The selection or adjustment of the alcoholic heart extract (before adding cholesterol) is also undertaken on a similar occasion.

The point that the constancy of the titrated positive controls depends upon the adjustments of the antigen-complement system was not made clear in the communication describing the titrated controls (Greval, Das and Sen Gupta, *loc. cit.*). The adjustments were not then discussed.

(10) *False and true variations in the serum reaction of a subject at different times.*

Reactions of the titrated positive controls vary when the necessary adjustments fail. When the adjustments are never made the reactions of certain sera in the tests proper are bound to vary too on repetition. How much of the variation is real can only be determined either by the behaviour of the titrated controls or by a flocculation test done at the same time.

True meteorologically conditioned variability of serological tests in syphilis (Hoverson *et al.*, 1935) has been reported. One of us (Greval, 1928) has also reported spontaneously occurring variations in the Wassermann and Kahn reaction of normal rabbits.

(11) *The incubator.*

The self-regulating water-bath does not prove satisfactory in localities like Calcutta where humidity is generally high and brass and copper surfaces turn green readily. An ordinary bacteriological incubator works better. As long as the titration and the test proper are all incubated in the same way, it is immaterial whether water or air surrounds the tubes.

The writers put up titrations and tests proper in $4'' \times \frac{1}{2}''$ test-tubes and incubate in an ordinary bacteriological incubator. Their racks are wooden holding one row of tubes. The racks are numbered and put together two deep for titration and five deep for tests proper.

(12) *Ammonia in the laboratory.*

Ammonia is known to damage the hæmolytic power of the complement (Gordon and Wormall, 1928). The vapour of ammonium sulphide is likely to be encountered in a high concentration in a room where blood stains are tested spectroscopically. It diffuses even into an adjoining room connected with a single communicating door. The writers minimize the concentration of the vapour (*i*) by keeping the number of preparation for spectroscopy minimal and (*ii*) by submerging the preparation in a 10 per cent solution of lead acetate after the spectroscopy.

VI. An important difference between the original technique and the modified techniques of the Wassermann reaction: a suggestion for a change of name of the reaction to lecithin complement fixation (L.C.F.).

The original technique of the reaction was materially different from the present techniques. According to Craig (1918) it depended upon the use of *a restricted quantity of the hæmolytic amboceptor* (2 *units*) *and an excess of the complement* (1 *c.c. of a* 1 *in* 10 *dilution, arbitrarily fixed*). In the tubes were placed the complement, 'the proper dose' of the antigen (not in the serum control), 0·2 c.c. of the patient's inactivated serum and enough saline to bring the total to 3 c.c. The tubes were incubated for one hour. Two units of amboceptor and 1 c.c. of 5 per cent corpuscle suspension were then added and the tubes incubated again until the serum controls were hæmolysed. The results were read at once or after several hours in the ice-box.

The present-day techniques of most workers in England and America depend upon the use of *an excess of the amboceptor and a restricted quantity of the complement.*

The original technique is one of a qualitative test and works fairly well as such. Quite a large number of workers on the continent of Europe plan their complement-fixation in general after the original technique, more or less. The later techniques are those of quantitative tests and give much more useful differentiations and readings which can be repeated with a fair amount of success. The fixation is measured in m.h.d. of complement and grades of positiveness assigned to the serum.

Some workers combine the two techniques. They use an excess of titrated hæmolytic amboceptor and also an excess of titrated complement. The results are read by restricting the time and by assigning degrees to the hæmolysis which is present in a large number of cases regarded positive. A special sensitiveness of the antigen is also relied upon. All these devices are unsatisfactory.

The worst technique is the one which uses an excess of titrated hæmolytic amboceptor and an excess of *untitrated* complement. In the tropics, where the titre of the complement varies from 1 in 30 or less in the hot weather to 1 in 90 or more in the cold weather, the results obtained by such a technique are useless even as results of a qualitative test.

The test in the techniques of which are employed standardized reagents and titrated controls is really so different from the original as to merit a change in name. In America the test has been re-named after the serologists who have introduced modifications. The present writers' suggestion is that the reaction should be re-named Lecithin Complement Fixation (L.C.F.). The name would be particularly useful in the tropics and in respect of cases elsewhere with tropical histories in allaying the alarm caused by doubtful, '50......90 per cent positive' and even positive Wassermann reactions. Diseases other than syphilis are undoubtedly responsible for the reaction in the tropics (Greval, Sen Gupta and Das, 1938).

The active substance in the antigen, presumed to be chiefly lecithin (Kolmer, *loc. cit.*), may be another allied lipoid or consist of several lipoids. The letter L will represent one or all of them.

Summary.

1. The quality of the complement with respect to the Wassermann reaction has been studied in this communication.

2. Optimal reaction and optimal titre of the complement are determined with the titration scheme of the method No. IV of the (British) Medical Research Council and titrated controls of pooled positive serum. A complement producing complete hæmolysis in the tubes of the same serial number in both the front and the hind row is a complement of optimal reaction. A complement m.h.d. of which is 1 in 40, 1 in 50 or 1 in 60 is a complement of optimal titre. The Wassermann reactions of the titrated controls with a complement of optimal reaction and titre and with a standardized antigen are typical.

3. Adjustments in the m.h.d. are made for a complement of optimal reaction but not of optimal titre: (*i*) for titre above 1 in 60 the reading is reduced by one tube and (*ii*) for titre below 1 in 40 an absolutely crystal clear tube is not insisted upon.

4. Complements of reactions other than optimal are: (*i*) cholesterol-shy complement and (*ii*) cholesterol-proof complement. For (*i*) mostly the cholesterol content of the antigen is reduced although at times complement is increased. For (*ii*) either the dose of the complement is reduced or phenol added to the antigen.

5. The reactions of the titrated controls help in reading the results of the test proper even when the above-mentioned adjustments have not been altogether successful. The adjustments which are nearly always successful (or successful enough for the purposes) are as necessary for borderline cases as the titrated amboceptor and complement and the standardized antigen.

6. The writers' other findings on complement and associated considerations are recorded. They consist of (*i*) further observations on cholesterol-shy and cholesterol-proof complement, (*ii*) preservation of complement, (*iii*) serum protein content of the complement and its m.h.d., (*iv*) anti-complementary sera, (*v*) effect of the magnitude and constancy of the m.h.d. on the test proper, (*vi*) delayed hæmolysis in the cold, (*vii*) two doses of the complement versus two dilutions of the serum, (*viii*) system of recording fixation, (*ix*) interdependence of titrations,

standardizations and adjustments, (*x*) false and true variations in the serum reactions of a subject at different times, (*xi*) incubation in air versus incubation in water, and (*xii*) dealing with ammonia vapour in the room.

7. In view of a very important difference between the original technique of the Wassermann reaction and the present-day techniques the suggestion is advanced that the reaction may now be re-named Lecithin Complement Fixation (L.C.F.).

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POSTSCRIPT.

Thompson and Maltaner (1940) have very recently further elaborated equations for the quantities of the antigens, antibodies and complement reacting in complement-fixation. They include complement-fixation for syphilis. The writers' opinion remains unchanged.—*S. D. S. G.*, 18th June, 1940.

Ind. Jour. Med. Res., **28**, 1, July, 1940.

THE FORM OF *PLASMODIUM GALLINACEUM* PRESENT IN THE INCUBATION PERIOD OF THE INFECTION.

BY

LIEUT.-COLONEL H. E. SHORTT, M.D., Ch.B., D.Sc., D.T.M. & H., I.M.S.,

K. P. MENON, L.M. & S., L.R.C.P. & S.,

AND

P. V. SEETHARAMA IYER, M.A.

(*From the King Institute of Preventive Medicine, Guindy, Madras.*)

[Received for publication, March 25, 1940.]

SINCE the discovery by James and Tate (1937) of the exo-erythrocytic development of *P. gallinaceum* the exact significance of these forms and of similar forms in other species of *Plasmodia* has been a source of speculation. This uncertainty has been increased by the fact that the exo-erythrocytic forms have been found present both in infections produced by the bites of mosquitoes and in those produced by blood inoculation. Their appearance in the latter case could only be by their origin from the erythrocytic pigment-producing forms or from forms of their own type in the circulating blood. The latter hypothesis is the one favoured by certain parasitologists, Raffaele (1938) for instance.

Up to the present all observers have stated their inability to find the exo-erythrocytic development before the appearance of pigment-bearing parasites in the circulation (James, 1938, 1939; Manwell and Goldstein, 1939), although James (1939) presumed, with reason, that the exo-erythrocytic schizogony was the normal type of early development of the parasite from the sporozoite stage.

On the other hand, it has been shown by inoculation of tissues during the incubation period in plasmodial infections. that some unrecognized form of parasite, capable of producing the disease, is present in various internal organs, although it does not enter the circulation (Warren and Coggeshall, 1937; Boyd and Matthews, 1939).

It is reasonable to suppose that these hitherto unrecognized forms are derived directly from sporozoites and represent a definite cycle of development leading to the production of forms capable of invading the erythrocytes. It is also a reasonable presumption, since their presence has been proved, that they have not been found because they are few in number when compared with the bulk of the organs, liver, spleen, bone-marrow and brain in which they may be contained.

On this assumption, we initiated experiments with *P. gallinaceum* intended to make the search for the 'incubation period' forms more easy by giving massive inoculations of sporozoites and using the smallest chicks available, viz., those still in the egg. We later found that the latter were not really necessary and that young hatched chicks were equally suitable.

Ædes ægypti or *A. vittatus*, preferably the former, were infected by feeding them on chicks infected with a Ceylon strain of *P. gallinaceum.* Ten to fourteen days later, the mosquitoes were dissected and the glands of those carrying sporozoites were used for inoculation into the test birds.

The sporozoites from one *A. ægypti* infected ten days previously were deposited on the chorio-allantoic membrane of a chick embryo thirteen days old and the membrane was scarified by the inoculating syringe needle. The egg was closed in the manner usual in this technique. Eleven days later the chick had made a hole in the egg-shell but had failed to emerge. On removal it was found dead. The post mortem revealed a most intense infection of the brain capillaries with exo-erythrocytic forms and these were also present in numbers in the liver, spleen and bone-marrow. The brain infection was the most intense we have seen. Smears from the heart blood revealed only a moderate infection of the blood with pigmented parasites.

This finding not only indicated that the chick was susceptible to infection with sporozoites while still in the egg, but the massive infection with exo-erythrocytic forms proved that they must have been developing for some days and that they would probably have been demonstrable even before the development of pigmented forms. This would mean that they were the long-looked-for incubation period stage of the parasite.

In our second experiment the sporozoites from an *A. ægypti* mosquito infected ten days previously were inoculated intramuscularly into a young chick about three weeks old. Seven days later the chick looked drowsy and was sacrificed. At the post mortem there was a well-developed infection with exo-erythrocytic forms in the brain and these were also present in liver, spleen and bone-marrow. Examination of the heart blood showed a few very young forms in the erythrocytes. These, although too young to show pigment, were evidently the beginning of the blood infection. Here again the infection of the brain with exo-erythrocytic forms was too heavy to be anything but the result of several days' development and it was, therefore, decided to examine chicks sooner after infection with sporozoites.

In a third experiment sporozoites from two *A. ægypti* mosquitoes infected twelve days previously were inoculated intramuscularly into a young chick about three weeks old. Six days later the chick was sacrificed. A very scanty infection,

of the brain only, with exo-erythrocytic forms was found. Careful examination of the blood showed no parasites in the erythrocytes*.

It would appear, therefore, that these were truly the forms of the parasite which develop during the incubation period and the assumption is that they derive directly from the sporozoites which, either as a result of their own aggressivity or of phagocytosis, find themselves in cells of the reticulo-endothelial system.

The exo-erythrocytic development presumably persists through a number of generations and goes on coincidentally with the erythrocytic development, at least for some time. James (1938) has shown that it is also responsible for the production of relapses and he concludes that it may arise *de novo* from the forms in the erythrocytes as well as from sporozoites. It does not seem to us necessary to accept this hypothesis. Having produced the forms which will establish the blood infection, it may continue to reproduce itself at a low level of activity hidden in the vast reservoir of the reticulo-endothelial system, ready to take on renewed activity when adequately stimulated, so producing relapses That it may so remain hidden without readily being demonstrated is illustrated by the fact that, so far as we know, no previous description has been given of these forms in the incubation period of the infection. Their demonstration now has been the result of relatively massive dosage of sporozoites.

Our conception of the course of events, based on a close study of tissues containing very large numbers of these exo-erythrocytic forms, is as follows, although, admittedly, it is merely an attempt to place in a proper sequence the many forms encountered.

It seems likely that the sporozoites inoculated by the mosquito either enter the blood directly or are carried into it via the lymph stream. The sporozoites are distributed throughout the body by the blood and are filtered out of the circulation by the cells of the reticulo-endothelial system and especially by the lining cells of the brain capillaries This latter location is somewhat curious because these cells are not normally phagocytic and it is possible that the sporozoites enter by their own efforts these cells which appear to be especially favourable to their development. The sporozoite may now be called a schizont. It increases in size and there appears to be a simultaneous increase in volume and division of nuclei until the volume of a large schizont may be 95μ by 7μ, or even larger, while the nuclei may be numbered by hundreds. The nuclei decrease in size as they increase in numbers and when schizogony is complete each merozoite consists of a small dot of chromatin with a scarcely perceptible accompaniment of cytoplasm. The schizont now presumably ruptures and the merozoites enter new cells to repeat the process. The process, therefore, up to this stage, is closely similar to the early stages of development among the coccidia. At a comparatively early stage in this series of generations some of the merozoites are destined to produce pigmented parasites and enter the erythrocytes thus initiating the erythrocytic cycle. With the establishment of the latter, however, the exo-erythrocytic cycle does not come to an end but appears to continue indefinitely, at least during the acute stages of

* This experiment has since been repeated.

the infection and, in fact, appears to reach its height at the period when the bird is either going to die of the infection or, gradually, to recover and become a bird with a chronic infection. It is for consideration whether the exo-erythrocytic cycle rather than the erythrocytic is not also responsible for the production of gametocytes. When the exo-erythrocytic cycle has apparently died down and is difficult or impossible visually to demonstrate it may still exist as a low grade development in the recesses of the reticulo-endothelial system and be responsible for keeping up the supply of gametocytes. If the exo-erythrocytic development is eventually found to be present in mammalian malaria this might be the explanation of chronic infections where the forms seen in the peripheral blood are sometimes almost exclusively gametocytes, a condition most often seen in malignant tertian infections. Relapses would thus appear to be due to a re-awakening of the exo-erythrocytic development resulting in due time in a new formation of erythrocytic elements such as occurs after the original invasion by sporozoites.

ACKNOWLEDGMENT.

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BABESIA SP. IN THE INDIAN LEOPARD, *PANTHERA PARDUS FUSCA* (MEYER).

BY

LIEUT.-COLONEL H. E. SHORTT, M.D., Ch.B., D.Sc., D.T.M. & H., I.M.S.

(From the King Institute of Preventive Medicine, Guindy, Madras.)

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A BLOOD slide taken from a leopard shot in South India in the Coimbatore district revealed a comparatively heavy infection with a species of *Babesia*. A search of such literature as was available failed to disclose any previous record of this genus in a leopard from any part of the world. This appeared somewhat surprising, considering the fact that the blood of many individuals must have been examined in zoological gardens throughout the world and it is possible that were the records of these available some mention of the genus would have been found. In any case, the presumption is that this parasite is not common and a short description is given below.

Only one blood slide was available for study and the description is based on this material alone.

The average diameter of the red blood cells of the leopard is $6{\cdot}1\mu$. The typical form of the parasite is a small ring like a minute malarial ring form. The chromatin body is relatively large in amount while the cytoplasmic portion of the ring is very delicate; the enclosed vacuole varies in size and determines the diameter of the parasite. Such forms are given in Plate VII, figs. 1 to 6, in which the chromatin body is seen to occupy varying proportions of the circumference of the parasite. Those forms in which the chromatin is elongated presumably represent early stages in division. The chromatin particle usually occupies a part of the circumference of the ring or is internally placed, seldom projecting externally. In some cases the chromatin would appear to occupy the whole circumference and in such forms it is difficult to distinguish the cytoplasm (Plate VII, figs. 7 and 8). In other cases only a small part of the circumference is composed of cytoplasm (Plate VII, figs. 9 to 11). Similarly, in the smallest forms, the chromatin body occupies the bulk of the parasite and the cytoplasm is barely indicated by the staining (Plate VII, figs. 10 and 11). The average size of ring forms of the parasite is $1{\cdot}32\mu$ but

extremes in size between 0·8μ and 2·0μ are met with, the largest forms usually being those in process of division.

Division forms.—A small granule appears to migrate to each end of an elongated chromatin body and this process may be seen in different stages of completion in Plate VII, figs. 12 to 16.

The cytoplasm separates into two bodies each tending to be somewhat pear-shaped (Plate VII, figs. 17 and 18) and these separate to form two daughter parasites (Plate VII, fig. 19) at which stage the pear shape is lost and the parasite assumes the ring form so that single pear-shaped forms are rarely seen. Division into more than two daughter forms, if it occurs at all, must be rare and was not encountered in the material available. It is presumed, therefore, that the two daughter parasites proceed to enter new red cells in order to repeat the process of binary fission.

Occasional forms, not corresponding closely to the description given, are sometimes seen, such as forms with a small detached dot of chromatin which may or may not be attached by a chromatin thread to the main chromatin mass (Plate VII, figs. 20 to 22) or forms with the cytoplasm elongated into a tag (Plate VII, fig. 23).

Identity of the parasite.—As already stated, no record of *Babesia* in leopards could be found and the morphology of the various species of *Babesia* is so similar that in the absence of full life-histories, including the identity of the carrier arthropod, the founding of new species is hardly justified. A search of the literature reveals certain recorded species which, from their descriptions, might all belong to one species if morphology alone were the criterion. Thus, the parasite would appear closely to resemble *B. civettæ* (Leger, 1920), also described by Wenyon and Hamerton (1930) and *B. felis* (Davis, 1929), but the average size seems to be much smaller.

Equally close is the resemblance to *B. bauryi* (*Nuttallia bauryi*) (Leger and Bedier, 1922) occurring in an African fox, *Fennecus dorsalis*, while a parasite of similar morphology has been noted in the African lion by the same authors. As in the case of the majority of recorded species of *Babesia* the extra-corporeal life-cycle in the arthropod host is not known. The Indian leopard always harbours large numbers of ticks of the genus *Hæmaphysalis* and it is hoped at some future date to examine these for stages in the life-cycle of this species of *Babesia*.

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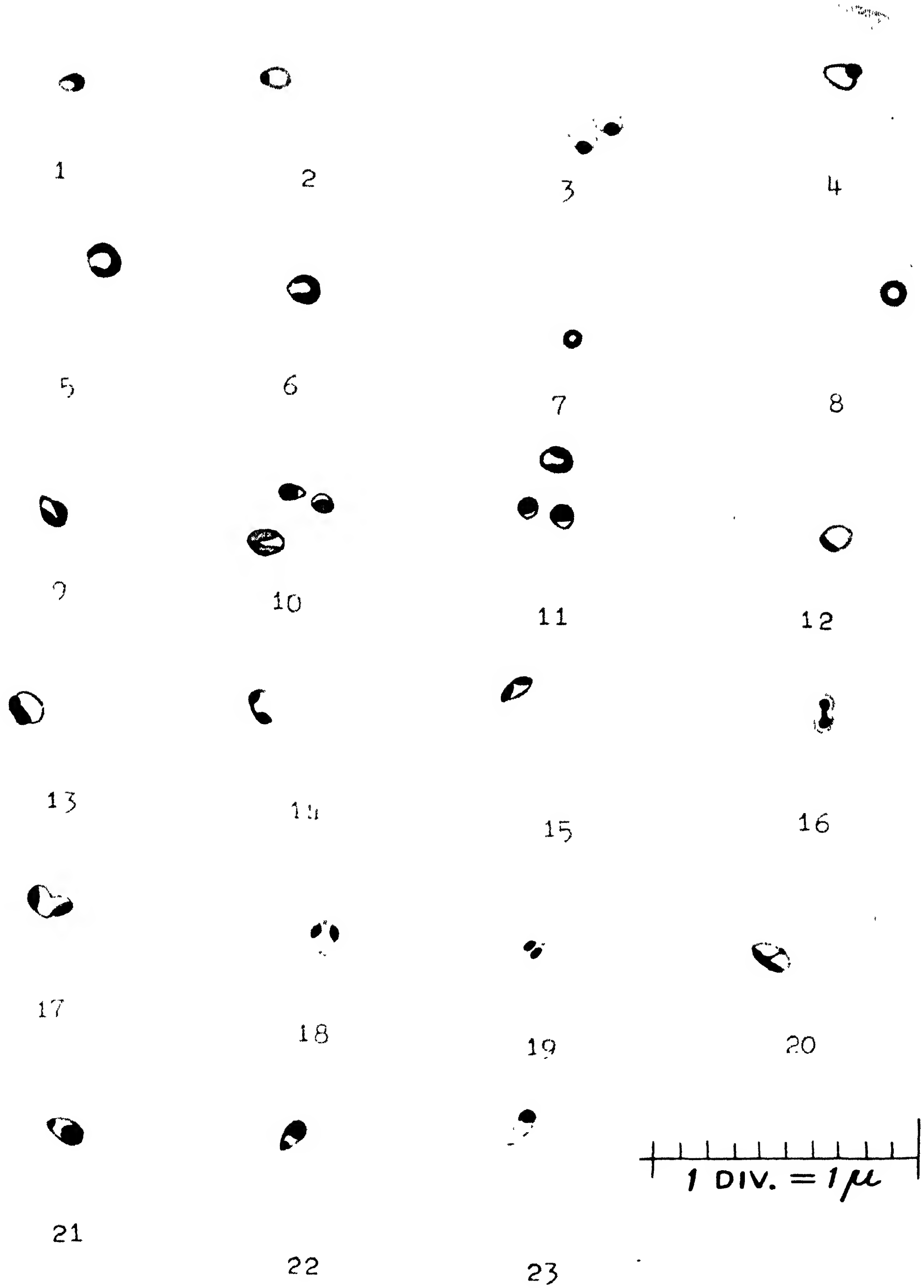
PLATE VII.



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OBSERVATIONS RELATIVE TO THE STANDARDIZATION OF COBRA ANTIVENENE.

BY

COLONEL J. TAYLOR, M.D., D.P.H., I.M.S.,
Director, Central Research Institute, Kasauli.

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No international standards yet exist for anti-venom sera and the methods of determining their potency which are employed by laboratories engaged in their manufacture vary widely. Ipsen (1938) in his progress report on the subject to the Permanent Committee of Biological Standardization, in which he deals with the methods in use for the estimation of potency of antivenenes for European viper venoms, shows that in four different laboratories the indications of potency taken are quite different. The experimental animals used are of three different species and the route of injection varies. The basis of estimation of potency used by the several laboratories dealt with are :—

1. Number of *certain lethal doses* (*d.c.l.*)* for the mouse neutralized by 0·1 c.c. of serum intravenously.
2. The quantity of serum neutralizing 1 mg. of venom for the guinea-pig subcutaneously.
3. The quantity of serum neutralizing 3·2 mg. of venom per kilogram weight of the rabbit intravenously.
4. The ratio of (dose of venom + 1 *d.c.l.*) to dose of serum for the mouse, intravenously.

No standard sera are used for control.

It is to be expected that the results obtained by these different methods would vary considerably and it is obvious that the toxicity of the particular sample of venom used in testing will directly affect the figure of potency assigned to any serum. Certain principles in determining the potency of antivenene are brought out in the course of the work on European viper venoms and the present investigation has been directed towards ascertaining their applicability to the standardization of cobra antivenene. The points which will be dealt with are :—

1. The suitability of intramuscular or intravenous routes of injection.

* The term *dosis certe lethalis* (*d.c.l.*) will be used throughout as this has been employed in reports of relative work quoted.

2. The relative toxicity of venom samples of different ages and their deterioration during storage.
3. The effect of testing potency of antivenene at different levels of venom dosage.

Experimental methods.

The venom used consisted of three batches of different ages each of which was composed of the product of the 'milkings' of the venom glands of a number of Indian cobras over a period of some weeks The venom had been dried over sulphuric acid and the resulting yellow granules were pooled in quantities of 5 to 10 grammes and stored in sealed test-tubes at room temperature. These can be considered to be average samples.

The method of standardization of cobra antivenene employed as a routine at the Central Research Institute, Kasauli, is by intramuscular injection of venom-antivenene mixtures in the pectoral muscles of pigeons but. for the purpose of the present investigations, it was decided to employ laboratory-bred white mice which can be used in larger numbers and are likely to be more regular in their reaction than mixed breeds of pigeons which are bought locally. The weight range selected was 18 to 20 grammes.

The question of route of injection was first investigated.

Toxicity of cobra venom by the intramuscular and intravenous routes.

The sample selected for test was venom A pooled in October 1937 and tested 1½ years later. Before use the venom was ground to a fine powder and dried to constant weight. Solutions were made up to contain the selected dose in a volume of 0·2 c.c. Injections were given intravenously in the tail vein of white mice in the usual way, and intramuscularly in the muscles at the inner side of the thigh close to the groin.

The following figures were obtained in the determination of the *certain lethal dose* by the two routes :—

Venom dose, mg.	Mortality.	Average hours to death.
1. *Intramuscular injection.*		
0·005	0/10	..
0·01	4/10	20½
0·0105	4/10	18
0·011	15/20	12
0·012	8/10	14
0·0125	10/10	13
0·015	10/10	6½

Venom dose, mg.	Mortality.	Average hours to death.
	2. *Intravenous injection.*	
0·005	1/10	9
0·006	8/20	9½
0·0065	4/10	18
0·0075	10/10	8
0·01	10/10	4¾

The results were on the whole more regular by the intramuscular route than by intravenous injection when mortality and death time are taken into consideration. With these doses no deaths occurred later than 24 hours, although in subsequent work, with higher multiples of the lethal dose partly neutralized, occasional but irregular deaths occurred at a later period. It was therefore decided to use for future work the intramuscular route in mice of 18 to 20 grammes weight with readings up to 24 hours.

Relative toxicity of different batches of cobra venom.

Using the same technique as described for intramuscular injections in white mice of 18 to 20 grammes weight three batches of cobra venom of different ages were tested :—

Venom A.—Pooled, October 1937. Tested, March 1939. Aged 1½ years. The results with this venom have already been given. The certain lethal dose (*d.c.l.*) was 0·0125 mg.

Venom B.—Pooled, July 1904. Tested, March 1939. Aged approximately 35 years. This was tested as above with the following results :—

Venom dose, mg.	Mortality.	Average hours to death.
0·011	2/10	23
0·012	2/10	24
0·013	2/10	12
0·0135	2/10	17
0·014	7/10	7½
0·015	10/10	7

The *d.c.l.* of this venom at time of testing was 0·015 mg.

Venom C.—Pooled, February-March 1939. Tested, late April 1939. Aged 1½ months.

Venom dose, mg.	Mortality.	Average hours to death.
0·0075	3/10	13
0·008	7/10	9½
0·009	10/10	7½
0·01	10/10	5

The *d.c.l.* of the venom was 0·009 mg.

In order of age of venoms the relative figures obtained for their *d.c.l.* were :—

			Mg.
Venom B, aged 35 years	..	..	0·015
,, A, ,, 1½ ,,	..	..	0·0125
,, C, ,, 1½ months	..	..	0·009

The venoms were subsequently kept in a finely ground state in a desiccator at room temperature and venoms B and C were re-tested later.

Tests of venom B made in December 1939, 9 months later, gave the following figures :—

Venom dose, mg.	Mortality.	Average hours to death.
0·015	..	..
0·016	2/10	15
0·017	6/10	15½
0·018	16/20	12½
0·019	10/10	12

The *d.c.l.* had increased from 0·015 mg. to 0·019 mg.

Venom C was used for neutralization tests, the details of which will be given later and the toxicity of control doses was observed over a period extending from July 1939 to January 1940.

In a series of tests made in July and August 1939 the combined figures for this venom were :—

Venom dose, mg.	Mortality.	Average hours to death.
0·0075	5/28	16
0·08	14/24	10½
0·09	26/27	7

In September 1939 only 80 per cent mortality was obtained with a dose of 0·009 mg. and the test dose had to be increased to 0·01 mg. in October, this dose producing 100 per cent mortality with a death time averaging 9¾ hours. Later in the same month the dose required to be raised to 0·011 mg. and in January the *d.c.l.* had increased to 0·012 mg.

Taking the freshest sample, venom C, aged 1½ months, as representing most nearly the toxicity of a fresh mixed sample it is seen that the difference between the *d.c.l.* of this sample and venom A, 1½ years old, is greater than that between venom A and the 35-year-old sample. It would appear probable that after a certain preliminary loss the venom has a considerable degree of stability when kept sealed up in the granular form. It should be noted that these venoms had been kept at room temperature only.

The loss in toxicity when ground up and kept in a desiccator is very marked and in the case of the oldest sample was probably greater in a few months than in a prolonged period of years as previously stored. Exposure to air in the finely ground state is probably responsible for this loss. Although the differences in toxicity of the samples are perhaps not so wide as might be expected on account of their relative ages, it is obvious that the use of the different venoms for standardization tests would result in marked differences in the findings on potency of antivenene.

Neutralization tests.

In order to ascertain the influence of the level at which tests of potency of antivenene are carried out a series of neutralization tests were made against multiples of 1 *d.c.l.* of cobra venom. The intramuscular route for injection in mice of 18 to 20 grammes was employed as in the case of the tests of toxicity and the results were recorded at 24 hours, deaths having been found to be irregular beyond that period. Mixtures of antivenene and venom solution were prepared which would provide a selected quantity of serum along with one or more *d.c.l.* of venom in a volume of 0·2 c.c. for each mouse. Contact for an hour at room temperature was allowed before injection.

Neutralization of 1 d.c.l.

Venom C was used for this test. The *d.c.l.* of this venom at the time of commencing the tests was 0·009 mg.

After a preliminary series of tests to obtain a general idea of the range for neutralization, in which difficulties were experienced owing to a gradual drop in toxicity of the venom when kept in the finely ground state, the following results were obtained with antivenene No. 12/38 :—

Venom dose, mg.	Antivenene, c.c.	Mortality.	Average hours to death.
	1		
0·01	0·0022	4/5	10
0·01	0·0024	3/5	15½
0·01	0·0026	0/5	..
0·01 (control)	..	10/10	9¾
	2		
0·01	0·0022	0/5	2 deaths at 31 hours.
0·01	0·0024	0/5	1 death at 35 hours.
0·01	0·0026	0/5	..
0·01 (control)	..	8/10	11½
(Toxicity had dropped and the control showed the dose to be insufficient).			
	3		
0·011	0·002	2/5	11½
0·011	0·0025	0/5	..
0·011	0·00275	0/5	..
0·011 (control)	..	10/10	11½

Further repeat tests confirmed the neutralizing dose of the antivenene for 1 *d.c.l.* of venom C to be 0·0025 c.c.

Neutralization of multiples of the d.c.l.

The neutralization of 2, 3, 4 and 5 *d.c.l.* of venom C by antivenene No. 12/38 was tested with final results which are summarized as follows :—

	Venom dose, mg.	Antivenene, c.c.	Mortality.	Average hours to death.
2 m. l. d. ..	0·022	0·0125	5/5	10¾
	0·022	0·015	1/5	13¾
	0·022	0·0175	0/5	..
	0·011 (control)	..	10/10	10¾
2 m. l. d. ..	0·022	0·0125	5/5	13½
	0·022	0·015	0/5	..
	0·022	0·0175	0/5	..
	0·011 (control)	..	10/10	8¾
3 m. l. d. ..	0·033	0·025	3/5	18½
	0·033	0·0275	0/5	..
	0·033	0·03	0/5	..
	0·033	0·0325	0/5	..
	0·011 (control)	..	10/10	11
4 m. l. d. ..	0·044	0·0375	3/5	14
	0·044	0·04	0/5	..
	0·044	0·0425	0/5	..
	0·011 (control)	..	10/10	11½
5 m. l. d. ..	0·055	0·05	2/5	12
	0·055	0·0525	1/5	21
	0·055	0·055	0/5	..
	0·011 (control)	..	10/10	11
5 m. l. d. ..	0·055	0·05	4/5	13½
	0·055	0·0525	2/5	21
	0·055	0·055	0/5	..
	0·011 (control)	..	10/10	11½

The proportions which the neutralizing doses of serum for one or more *d.c.l.* of the venom bear to each other are indicated by the following figures :—

Number of *d.c.l.*	Quantity of serum required to neutralize.
1	0·0025 c.c.
2	0·015 c.c. = 0·0025 c.c. + 0·0125 c.c.
3	0·0275 c.c. = 0·0025 c.c. + (0·0125 c.c. × 2).
4	0·04 c.c. = 0·0025 c.c. + (0·0125 c.c. × 3).
5	0·55 c.c. = 0·0025 c.c. + (0·0125 c.c. × 4).

For every additional *d.c.l.* of venom above 1 *d.c.l.* five times the quantity of serum neutralizing 1 *d.c.l.* has to be added to obtain neutralization. Taking the quantity of serum neutralizing 1 *d.c.l.* as unity the amounts required for 2, 3, 4 and 5 *d.c.l.* are 1 + 5, 1 + 10, 1 + 15 and 1 + 21.

While this proportion was preserved throughout the range tested, with a slight variation at 5 *d.c.l.* neutralization was not accomplished with the same accuracy at the level of 10 *d.c.l.*

With 10 *d.c.l.* the results when a corresponding proportion of antivenene to venom was employed were :—

Venom dose, mg.	Antivenene, c.c.	Mortality.	Average hours to death.
0·11	0·1125	2/5	21
0·11	0·115	4/5	21¼
0·11	0·1175	4/5	21¼
0·011 (control)	..	10/10	13½

The death time was somewhat prolonged and the mice surviving at 24 hours died later up to 48 hours. So small a difference from a very exact *d.c.l.* as 0·0005 mg. when multiplied to produce 10 *d.c.l.* would possibly account for this.

In order to confirm this relationship between the amounts of antivenene required to neutralize single and multiple *d.c.l.* repeat tests were carried out with

a serum of lower potency and with venom showing a *d.c.l.* of 0·012 mg., and the following results were obtained :—

Venom dose, mg.	Antivenene, c.c.	Mortality.	Average hours to death.
	1		
0·012 (1 *d.c.l.*) ..	0·0025	5/5	10¼
0·012 („) ..	0·0035	0/5	..
0·024 (2 *d.c.l.*) ..	0·015	3/5	8¼
0·024 („) ..	0·021	0/5	..
0·012 (control) ..	..	10/10	10½
	2		
0·012 (1 *d.c.l.*) ..	0·003	2/5	16
0·012 („) ..	0·0035	0/5	..
0·024 (2 *d.c.l.*) ..	0·018	1/5	24
0·024 („) ..	0·021	0/5	..
0·012 (control) ..	..	10/10	8

In these tests also the additional amount of antivenene required to neutralize an extra *d.c.l.* was five times that neutralizing one initial *d.c.l.*

Tests were also carried out in pigeons of 300 grammes weight by the injection of venom-antivenene mixture in a volume of 1 c.c. in the pectoral muscles. The *d.c.l.* of the venom used was ascertained to be 0·2 mg. Neutralization of 1 *d.c.l.* and 2 *d.c.l.* only was tested.

Venom dose, mg.	Antivenene, c.c.	Mortality.	Average hours to death.
0·2 (1 *d.c.l.*) ..	0·04	2/3	9
0·2 („) ..	0·05	1/3	10½
0·2 („) ..	0·06	0/3	..
0·2 („) ..	0·07	0/3	..
0·4 (2 *d.c.l.*) ..	0·24	2/3	15½
0·4 („) ..	0·30	1/3	21
0·4 („) ..	0·36	0/3	..
0·2 (control) ..	..	3/3	13½

For pigeons also the same proportion between the amounts of antivenene neutralizing 1 *d.c.l.* and 2 *d.c.l.* was found to obtain, these being respectively 0·06 c.c. and 0·36 c.c.

The findings in regard to the relative amounts of cobra antivenene required to neutralize 1 *d.c.l.* and its lower multiples show a marked correspondence with those of Banic and Ljubetic in the case of the venom of the European viper *ammodytes*. Ipsen (*loc. cit.*) in summarizing this work says, 'These investigators have observed that the amount of serum required to neutralize 2 *d.c.l.* is much larger than twice the amount neutralizing 1 *d.c.l.* The authors explain this by assuming that a large part of 1 *d.c.l.*, say four-fifths, is neutralized by the natural defences of the animal's organism. Only the remaining fifth of 1 *d.c.l.* requires neutralization by the serum to protect the animal. When 2 *d.c.l.* is given to the animal much more serum is required—namely, the amount neutralizing the supposed fifth of 1 *d.c.l.* + the amount of serum neutralizing a full *d.c.l.*'

Banic and Ljubetic's figures show that approximately five times the amount of serum neutralizing 1 *d.c.l.* of *ammodytes* venom is required to neutralize 2 *d.c.l.* and that this proportion also obtains in higher multiples. The figure for cobra antivenene is six times, the route of injection being intramuscular instead of intravenous and the nature of venom action quite different.

It is evident that the calculation of the neutralizing value of an antivenene should not be based on the amount required to neutralize 1 *d.c.l.* but on the amount neutralizing additional lethal doses, to eliminate the factor of natural resistance of the test animal's organism. The method of calculation applied to Banic and Ljubetic's results by Ipsen appears to be a suitable one for estimating the potency of a serum.

The formula is :—

$$\left.\begin{array}{c}\text{Amount of venom neutralized}\\ \text{by 1 c.c. of antivenene}\end{array}\right\} = \frac{\text{Number of } d.c.l. - 1\ d.c.l.}{\text{Dose of serum} - \text{dose (S)}}$$

(where S is the quantity protecting against 1 *d.c.l.*).

On this basis 1 c.c. of cobra antivenene No. 12/38 would be calculated as neutralizing 80 *d.c.l.* for the white mouse or 0·88 mg. of venom C, either the number of lethal doses or the actual weight of venom used being applied in the formula.

As the proportion found between the amount of antivenene neutralizing 1 *d.c.l.* and 2 *d.c.l.* or other low multiples of cobra venom is a regular one and has been found to obtain in pigeons as well as in white mice the figure for the true neutralizing value of a cobra antivenene could be taken as five times the quantity protecting against 1 *d.c.l.* and calculation on the formula would not be necessary in this case.

Discussion.

It is for consideration whether the principle employed in determining the potency of an anti-toxic serum by comparison with a 'standard serum', a certain quantity of which is adopted as a 'unit', should necessarily be applied to the

standardization of anti-venom sera. The principle is a suitable one in the case of anti-toxic serum as the lethal dose of a toxin against which tests are carried out is minute and the toxins obtained in culture vary very widely in their potency. In addition the amount of toxin to be neutralized in the therapeutic treatment of any individual case by means of an antitoxin is unknown and the dose given, in arbitrary units, is based only on clinical experience and observations.

On the other hand, a venom such as that of the cobra has a weighable lethal dose and the toxicity of different samples of the venom varies within comparatively narrow limits. The maximum amount of venom which a snake of a particular species, such as the Indian cobra, may inject and the average dose given and the lethal dose for man has been worked out in terms of milligrams of dried venom. The most suitable indication for the practitioner in determining the dosage of antivenene to be given in a case of snake bite would appear to be the definition of the potency of a serum in terms of the amount of venom which it is able to neutralize. The introduction of the use of an arbitrary unit of potency seems unnecessary and would be confusing.

A ' standard serum ' for cobra antivenene with an arbitrary unitage has been introduced in the Union of South Africa. As shown by Finlayson and Grobler (1939), who have employed methods of testing exactly analogous to those employed in the testing of anti-toxic sera, the use of the standard enables a control to be made of the relative potency of different commercial antivenenes. The method of estimation of potency, although apparently satisfactory in practice, is an indirect one.

As has been shown, the range within which the toxicity of cobra venom, under different conditions of storage, may vary is not very wide but it is sufficient to introduce irregularities in the results of testing and, as Ipsen (*loc. cit.*) concludes, the relative potency of two or more sera is only constant when standardization is carried out against the same venom sample. It is possible to devise methods of storing dried snake venom which would result in maintenance of their toxicity as a steady level but the distribution of a ' standard venom ' against which tests of potency may be carried out would present certain difficulties.

Ipsen has suggested that it may be advisable, in the case of European antiviper sera, to establish standard venom preparations and that the potency of the sera might be expressed in reference to these preparations. If the determination of the potency of an antivenene is carried out on the basis of number of *d.c.l.* neutralized it would not be necessary to carry out testing against the standard venom itself. Testing could be carried out against any homologous venom sample and the result calculated on the basis of the figure of toxicity of the standard preparation. For example, if cobra venom C at the time of its first examination when its *d.c.l.* was 0·009 mg. was selected as the standard venom, an antivenene neutralizing 80 *d.c.l.* per c.c. when the *d.c.l.* of the venom used in testing was 0·011 mg. would be calculated as neutralizing 0·72 mg. per c.c. and not 0·88 mg.

It is of interest to note that the basis of calculation suggested, if applied to the results of the neutralization of a cobra venom sample with a *d.c.l.* of 0·012 mg. in

white mice and to tests in pigeons with the same venom gives corresponding results. The figures were :—

White mouse	..	0·0175 c.c.	neutralizes	0·012	mg.
		1·0 c.c.	,,	0·68	,,
Pigeon	..	0·3 c.c.	,,	0·2	,,
		1·0 c.c.	,,	0·66	,,

Summary.

1. On account of differences found in the toxicity of different samples of cobra venom which may be available for testing the potency of antivenene it is suggested that assay should be carried out on the basis of the number of 'certain lethal doses' neutralized by a given quantity of serum.

2. If a figure of *d.c.l.* is established for the selected test animal of given weight by the chosen route which is taken as the 'standard toxicity' of the venom of the species the results could be expressed in terms of weight of standard toxin neutralized.

3. The observations made on the neutralization of 1 *d.c.l.* and its multiples show the necessity of eliminating the effect of natural resistance of the test animal's organism in calculating the potency of a serum. The formula applied by Ipsen (*loc. cit.*) to Banic and Ljubetic's findings appears to be a suitable basis of calculation. A simpler method of calculation is given on the basis of proportion found to exist between the amount of antivenene required to neutralize 1 *d.c.l.* and its multiples.

4. The opinion is expressed that the direct estimation of the potency of cobra anti-venom serum in terms of the weight of dried venom neutralized per cubic centimetre is more suitable than assay in terms of a 'standard serum' with arbitrary unitage.

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Finlayson, M. H., and Grobler, J. M. (1939). .. *South Afr. Med. Jour.*, **13**, No. 1, p. 9.

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NOTICE

In future the Volume of the *Indian Journal of Medical Research* will be issued to correspond with the calendar year. The present Volume, No. **28**, will terminate with the issue of October 1940. Volume **29** will consist of four quarterly numbers commencing with the January 1941 issue. The change is being made for convenience of reference.

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THE BLOOD IN CHOLERA.

Part I.

TECHNICAL METHODS.

BY

K. S. MALIK,

AND

MAJOR C. L. PASRICHA, I.M.S.

(From the Cholera Bacteriological Inquiry, Indian Research Fund Association, School of Tropical Medicine, Calcutta.)

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OWING to the difficulty of collecting large samples of blood from the collapsed veins of cholera patients during the acute stage of the disease it was necessary to use methods in which small quantities of blood would suffice for the estimation of certain constituents. It was possible to make a number of estimations with 6 c.c. of blood which amount can be collected with comparative ease from the majority of cholera patients. The estimations made and the amount of blood required for each estimation are shown in Table I in which the different estimations are arranged in the order they were made. In Table II are given the tests made with the available plasma.

The methods used and found satisfactory are given in this Part and the results obtained in 17 cholera patients in Part II of this series (*see* page 301 *et seqq.*).

The anticoagulant used is potassium oxalate in proportion of 3 mg. of potassium oxalate for each cubic centimetre of blood. A 30 per cent solution of potassium oxalate is prepared and the required amount of this stock solution (0·01 c.c. for each c.c. of blood) is added to small Pyrex tubes, spread on the sides of the tubes and dried *in vacuo* over calcium chloride.

The blood is collected from a vein, put into an oxalate tube, rotated to mix the anticoagulant and immediately taken to the laboratory. It is convenient to

have ready sets of tubes containing anticoagulant sufficient for 4 c.c., 5 c.c. and 6 c.c. of blood in case the required amount of blood (6 c.c.) is not obtained.

TABLE I.

The estimations made with the whole blood and the amount of blood used.

Estimation.	Amount of blood in c.c. required for the test.
Cell volume * (in duplicate) ..	4
Sugar (whole blood) ..	0·10
Hæmoglobin	0·025
Moisture	0·25
Urea	0·50
Chlorides (expressed as NaCl) ..	0·10

* Cell volume was tested in duplicate and the plasma collected from the cell-volume tubes used for the estimation of the plasma constituents.

TABLE II.

The estimations made with the available plasma (collected from the cell-volume tubes and the remaining 1 c.c. of blood).

Estimation.	Amount of plasma required in c.c.
Sugar (plasma)	0·10
Total non-fibrin nitrogen ..	0·20
Globulin	0·10
Moisture	0·25
Chlorides (expressed as NaCl) ..	0·10
Non-protein nitrogen and inorganic phosphate.	0·50

(*i*) *Cell volume.*—About 2 c.c. of the oxalated blood is transferred to two graduated cell-volume tubes. The tubes are centrifugalized at 3,500 r.p.m. for

half an hour and the volume of packed red blood cells noted. Corrections for any possible shrinkage of cells due to the presence of potassium oxalate were not made. It has been shown by Eisenman (1927) and other workers that a shrinkage of 1·5 to 2 per cent in the cell volume occurs when potassium oxalate is used as the anticoagulant, but Ponder (1934) in a critical examination of this subject demonstrated that shrinkage of cells does not occur with potassium oxalate and that the higher volumes obtained by workers with heparin as the anticoagulant were due to the development of tiny clots in the heparinized blood.

(*ii*) *The glucose present in the whole blood and in the blood plasma* (*removed from the cell-volume tube*).—For this purpose 0·1 c.c. of blood or 0·1 c.c. of plasma is used. Hagedorn and Jensen's method as quoted by Cole (1933) is employed. The 0·1 N NaOH and 0·45 per cent zinc sulphate are freshly prepared from stock solutions of 2 N sodium hydroxide and 45 per cent zinc sulphate. The values for glucose are calculated from the table given by Peters and van Slyke (1932).

(*iii*) *Hæmoglobin.*—The hæmoglobin percentage is estimated by the Newcomer's method using the Klett-Newcomer glass standard equivalent to a 0·038 per cent hæmoglobin solution. Hundred per cent hæmoglobin by this method is equivalent to 15·3 grammes hæmoglobin.

(*iv*) *Moisture content of blood and plasma.*—Approximately 0·25 c.c. of blood or plasma is spread on a piece of thick dry filter-paper and the amount of moisture determined according to the Bang's method as quoted by Obermer and Milton (1935).

(*v*) *Blood urea.*—For this estimation 0·5 c.c. of the blood is de-proteinized by Haden's (1923) modification of the Folin and Wu method. The urea is estimated in the protein-free filtrate by a modification of the xanthydrol colorimetric method of Beattie (1928). The modification employed is that after precipitation of the urea as dixanthyl urea from the protein-free filtrate and from the standard urea solution in the centrifuge tubes, the precipitate is not filtered off by suction through a Gooch crucible but centrifuged at about 3,000 r.p.m. for 15 minutes after which the supernatant fluid is decanted. The residue is washed with 5 c.c. of methyl alcohol saturated with dixanthydryl urea. This washing is repeated and a final washing is made with 2 c.c. of methyl alcohol. The precipitate is dried by inverting the centrifuge tubes over filter-paper and allowing it to stand at room temperature for about an hour. The colour is then developed with 50 per cent sulphuric acid and estimated colorimetrically.

(*vi*) *Blood and plasma chlorides.*—For the estimation of blood and plasma chlorides a micro-method was used. This is a modification of the well-known open Carius' method in which the estimation of the chlorides can be made in 0·1 c.c. of the blood or plasma. The details of the micro-method are given below :—

(*a*) *Reagents required* :—

1. *Silver nitrate* (0·02 N) in pure nitric acid. 0·6796 gramme of silver nitrate (guaranteed reagent grade) is dissolved in a minimal amount

of water and the volume made up to 200 c.c. with concentrated nitric acid (specific gravity 1·40, halogen-free guaranteed reagent grade). This solution keeps well if kept in a dark bottle.

2. *Ferric nitrate solution.*—Dissolve 4 g. of ferric nitrate in about 90 c.c. of water and make up to 100 c.c. with concentrated nitric acid (specific gravity 1·40, halogen-free guaranteed reagent grade). This solution is colourless.

3. *Potassium sulphocyanate solution.*—An approximately centinormal solution is made by dissolving 0·5 g. of the guaranteed reagent grade salt in 500 c.c. distilled water.

4. *Potassium permanganate* 6 per cent solution.

5. *Acetone.*

6. *Oxalic acid crystals.*

(*b*) *The micro-determination of chlorides.*—With a 'to deliver' pipette add 0·10 c.c. of blood or plasma to a small Pyrex test-tube (4″ × $\frac{3}{4}$″) and then 1 c.c. of 0·02 N silver nitrate solution adding drop by drop and shaking after the addition of each drop. Warm the mixture and gradually bring to a gentle boil. The proteins dissolve and the solution becomes clear with a precipitate of silver chloride at the bottom. To aid and accelerate the digestion of the proteins and other organic matter add the 6 per cent potassium permanganate solution a drop at a time, boiling in between till the solution becomes almost colourless and the permanganate colour after the addition of the last drop persists for about half to one minute. Decolorize any excess of potassium permanganate with a small crystal of oxalic acid. Keep boiling gently to decompose any silver oxalate that may have formed, to drive off any nitrous acid fumes and also to reduce the amount of liquid in the tube. Add a few drops of freshly boiled nitric acid (free from nitrous acid) and a drop of the ferric nitrate solution. Cool the tube in iced water and add 3 c.c. of ice-cooled acetone, washing the sides of the tube with it. Separate the silver chloride precipitate by decanting or, if necessary, centrifuge the tube and transfer the supernatant liquid to another (4″ × $\frac{3}{4}$″) Pyrex test-tube. Wash the precipitate with 1 c.c. of acetone shaking the tube vigorously. Decant or centrifuge and transfer the supernatant to the first washing. Cool the washings thoroughly in ice-cold water. The end-point is sharper at low temperatures. Titrate with 0·01 N potassium sulphocyanide till the first appearance of a light pink colour which persists for about half a minute. Perform a blank titration using 1·0 c.c. of the standard silver nitrate solution without the addition of blood.

Note.—(1) It is important to cool the tube before adding acetone, otherwise nitrous acid fumes are given which colour the solution and it is difficult to obtain a sharp end-point.

(2) In order to get accurate results silver chloride must be removed from the solution to be titrated. If the silver chloride is not removed the sulphocyanide reacts with it forming the more insoluble silver sulphocyanide and setting free the chloride ions. Further, if the silver chloride is not removed it reacts with the ferric

sulphocyanide forming silver sulphocyanide and ferric chloride with the result that a sharp end-point is not obtained.

Calculation :—

$$\frac{A - B}{A} \times C \times 1{,}000 = \text{mg. sodium chloride per 100 c.c. of blood.}$$

A = the volume of the sulphocyanide solution required to titrate 1 c.c. of the 0·02 N silver nitrate standard solution—the 'blank' titration.

B = the amount of sulphocyanide required to titrate the excess of the 1 c.c. of the standard silver nitrate solution after its reaction with 0·1 c.c. of blood.

C = the amount of sodium chloride equivalent to 1 c.c. of the standard 0·02 N silver nitrate.

The moisture, glucose, and the chloride content of the plasma were estimated by the methods used for the whole blood. The estimation of total nitrogen, the non-fibrinogen nitrogen and the non-globulin nitrogen in the plasma is detailed below.

The plasma albumin was calculated by taking the difference between the total plasma nitrogen and the sum of globulin nitrogen and fibrin nitrogen. As the amount of plasma available for these tests was small micro-methods were used for these estimations.

Reagents :—

(*a*) *Sodium chloride* 0·8 per cent.

(*b*) *Concentrated sulphuric acid* saturated with copper sulphate.

(*c*) *Sodium sulphate* 22·2 per cent solution.

(*d*) *Calcium chloride* 2·5 per cent solution.

(*e*) *Standard ammonium sulphate solution* containing 1 mg. of nitrogen per 10 c.c. of solution.

(*f*) *Nessler's solution.*

Dilution of the plasma.—With a 'to contain' pipette deliver 0·20 c.c. of plasma into a tall 5-c.c. measuring cylinder containing about 3 c.c. of 0·8 per cent sodium chloride. Rinse pipette carefully and finally wash it with saline from the top. Make up with saline to 4-c.c. mark. If there are any bubbles in the diluted plasma that interfere with accurate measurement these can be conveniently removed by holding a hot platinum loop near but not touching the surface.

(*vii*) *Estimation of total nitrogen.*—Into a Pyrex test-tube (6″ × ¾″) place 0·5 c.c. of the diluted (1 in 20) plasma and add 0·4 c.c. of concentrated sulphuric acid saturated with copper sulphate. Put two small glass-beads in the test-tube to prevent bumping. The proteins are digested by heating the test-tube gently over a micro-burner till white fumes begin to appear. At this stage cover the mouth of the test-tube with a pear-shaped glass-bulb to prevent the loss of fluid during the subsequent heating. In a few minutes the solution becomes light

brown in colour and when this occurs add a drop of H_2O_2 (perhydrol) and continue heating till the fluid becomes clear with a light greenish blue tinge. Usually one drop of perhydrol is sufficient, but if the digestion has not been completed more can be added. It is important that any particles of carbonaceous matter that have crept up the side of the tube be brought back into the body of the fluid by careful tilting of the tube. Allow the tube to cool and, when cool, add about 10 c.c. of water to dissolve the digested material and pour the resulting solution into a 50-c.c. measuring flask. Wash the tube several times with water and add the washings to the flask and make up the volume to 50 c.c. with distilled water. Transfer 25 c.c. of this solution to an empty 50-c.c. measuring flask, add 15 c.c. of water, shake and add 6 c.c. of Nessler's solution with a wide-mouth delivery pipette. Mix the contents well and make up to 50 c.c. with distilled water. Make the standard solution as follows :—

	c.c.
Standard ammonium sulphate solution	2
Water	40
Concentrated sulphuric acid saturated with copper sulphate ..	0·2
Nessler's solution	6
Water to	50

Compare the unknown with the standard in the colorimeter.

There should be no cloudiness after the addition of the Nessler's solution. If cloudiness occurs it is due to one of the following :—

(1) Low alkalinity of the original Nessler's solution.

(2) The too slow addition of Nessler's solution or inadequate mixing of the solution.

(3) Use of an excess of concentrated sulphuric acid.

(4) Incomplete digestion of the plasma proteins.

(5) Traces of acetone in the pipettes (from the acetone used in drying the pipettes).

(6) A delay in making the final comparisons. The reading should be taken within half an hour of the addition of Nessler's solution.

Calculation :—

Reading of standard = S.

Reading of solution under test = R.

$$\text{Total nitrogen} = \frac{S}{R} \times \text{strength of S} \times \frac{\text{dilution of R}}{\text{dilution of S}} \times 2 \times \frac{100}{0{\cdot}025}.$$

(*viii*) *Estimation of non-fibrin nitrogen.*—Into a Pyrex test-tube (4″ × ¾″) add the following :—

	c.c.
Plasma diluted (1 in 20 as above)	3
Sodium chloride (0·8 per cent solution)	2·8
Calcium chloride (2·5 per cent solution)	0·2

Mix well and cork tightly with a paraffined cork. Keep for an hour at 37°C. in an incubator (preferably in a moist incubator). At the end of an hour insert into the clot a thin glass-rod so as to reach to the bottom of the clot and rotate the glass-rod briskly. If done carefully the fibrin sticks to the rod and can be removed with the rod. The fluid is filtered through filter-paper (Whatman No. 3, 5·5 cm. in diameter). The filtrate should be crystal clear.

In order to avoid the loss of fluid by evaporation it was found advisable to arrange the filtration in a closed glass-jar such as a desiccator with a layer of water at the bottom. In this way the filtration is carried out in a humid atmosphere.

The non-fibrin nitrogen in 1 c.c. of the filtrate (representing 0·025 c.c. of the original plasma) is estimated in the same way as in the estimation of total nitrogen described above.

(*ix*) *Estimation of non-globulin nitrogen.*—Into a Pyrex tube ($4'' \times \frac{3}{4}''$) add the following :—

		c.c.
Plasma (undiluted)	.. ..	0·10
Sodium sulphate (22·2 per cent solution)	.. ..	3

Shake to mix thoroughly and cork the tube with a paraffined cork. Keep the tube in an incubator at 37°C. for three hours (preferably in a moist incubator). Filter (Whatman filter-paper No. 3, cut to 3·5 cm. in diameter) using the filtering device described above to avoid loss of fluid by evaporation during the process of filtration. The filtrate should be crystal clear.

The nitrogen in 1 c.c. of the filtrate is estimated as in the estimation of total nitrogen described above. The non-globulin nitrogen is calculated from the following :—

$$\frac{S}{R} \times \text{strength of } S \times \frac{\text{dilution of } R}{\text{dilution of } S} \times 62 \times 100.$$

(*x*) *Estimation of plasma non-protein nitrogen.*—This is carried out as follows :—

The de-proteinization of plasma.—With an Ostwald pipette add 0·5 c.c. of undiluted plasma to 3·5 c.c. of distilled water contained in a Pyrex centrifuge tube. Mix and add 1 c.c. of 30 per cent trichloracetic acid. Cork with a paraffined cork and mix contents thoroughly by shaking. Leave on bench for half an hour and then centrifuge. Filter the supernatant fluid through Whatman filter-paper (No. 1, 3·5 cm. in diameter) using the filtering device described above. If done with care it is possible to obtain a little over 4 c.c. of filtrate. A drop of the filtrate is mixed with 20 per cent sulphosalicylic acid to make certain that the filtrate is protein-free.

Take 2 c.c. of the filtrate as prepared above in a ($6'' \times \frac{3}{4}''$) Pyrex test-tube, add 2 glass-beads and concentrate the filtrate by heating at a temperature of 110°C. to 120°C. (in a sulphuric acid-bath).

When about 0·5 c.c. of the liquid remains, cool the tube and add 0·2 c.c. of concentrated sulphuric acid saturated with copper sulphate and digest on a micro-burner. Add a drop of H_2O_2 (perhydrol) to complete the digestion. Dissolve in water and make up with water to 20 c.c. in a 20-c.c. measuring flask.

Transfer 10 c.c. of this digested solution to 25-c.c. measuring flask, add 10 c.c. of water, mix and add 3 c.c. Nessler's solution and make up to 25 c.c. with distilled water.

Compare in the colorimeter with a standard solution made up as follows :—

	c.c.
Standard ammonium sulphate	0·5
Digesting sulphuric acid	0·1
Water	20
Nessler's solution	3
Make up with water	25

Calculation :—

$$\text{N.P.N.} = \frac{S}{R} \times \frac{\text{dilution of R}}{\text{dilution of S}} \times 0{\cdot}05 \times \frac{100}{0{\cdot}2} \times 2.$$

(*xi*) *The estimation of inorganic phosphates.*—This estimation was made by a modification (employing smaller quantities) of the method described by Youngberg and Youngberg (1930). The reagents used for the estimation of phosphorus are the same as used by Youngberg and Youngberg and are as follows :—

(*a*)	Sulphuric acid solution	10 N.
(*b*)	Molybdate sulphuric acid mixture—	c.c.
	Sodium molybdate (7·5 per cent)	50
	Sulphuric acid (10 N)	25
	Distilled water	25
(*c*)	Stannous chloride solution—	
	Stannous chloride	10 g.
	Hydrochloric acid (concentrated)	25 c.c.

Store in a brown glass-stoppered bottle.

(*d*)	Standard phosphate solution (10 c.c. = 1 mg. phosphorus)—	
	Mono-potassium phosphate (KH_2PO_4)	0·4394 g.
	Water	1,000 c.c.

Add a few drops of chloroform to prevent the formation of moulds and keep well stoppered. For each day's work prepare fresh dilutions of the following standard stock solution :—

1. Dilute the stannous chloride solution 1 in 500 by taking 0·05 c.c. of the stock stannous chloride solution and making up to 10 c.c. with distilled water.

2. Dilute the standard potassium phosphate solution 1 in 10. One cubic centimetre of this solution is equal to 0·01 mg. phosphorus.

Take in a 10-c.c. measuring flask 1 c.c. of the protein-free filtrate of plasma (prepared after precipitation of proteins with trichloracetic acid as described in the method of estimation of non-protein nitrogen). Add 7 c.c. of water, 1 c.c. of the molybdate sulphuric acid mixture and 0·5 c.c. of the diluted stannous chloride solution and make up to 10 c.c. In another 10-c.c. measuring flask prepare a similar mixture using 1 c.c. of the dilute standard phosphate solution instead of the protein-free filtrate of plasma. Compare in the colorimeter.

Calculation :—

$$\frac{S}{R} \times 10 = \text{mg. phosphorus per 100 c.c. plasma.}$$

Summary.

The methods used for the estimation of certain constituents in the blood of cholera patients and found to give satisfactory results are described.

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THE BLOOD IN CHOLERA.

Part II.

CERTAIN CHEMICAL CONSTITUENTS.

BY

MAJOR C. L. PASRICHA, I.M.S.,

AND

K. S. MALIK.

(From the Cholera Bacteriological Inquiry, Indian Research Fund Association, School of Tropical Medicine, Calcutta.)

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IN this Part of the paper are given the results of the estimation of certain chemical constituents in the blood of 17 cholera patients. The blood was collected within 24 hours of the onset of the disease and before the administration of any salines. At the time of the collection of blood all the patients were approximately in the same stage of the disease, the acute stage with imperceptible pulse, cyanosed with hurried respiration and scanty or suppressed urine. The clinical diagnosis was confirmed subsequently by the isolation of *Vibrio choleræ*. Except patient No. 2 who died on the first day of the disease all the patients recovered rapidly and were discharged well in 7 to 10 days after admission. They were all adults drawn from approximately the same strata of society—the lower working class.

The blood was collected from the veins into a tube containing potassium oxalate and sent immediately to the laboratory. The estimations were made soon after the collection of the sample. The cell volume, hæmoglobin, blood and plasma moisture, blood and plasma glucose, blood and plasma chlorides, blood urea, plasma non-protein nitrogen and plasma inorganic phosphates, the plasma proteins and the fractions of albumin, globulin and fibrin were estimated. The methods employed have been summarized in Part I of this paper (Malik and Pasricha, 1940) (*see* page 291 *et seqq.*). The results are given in Table I :—

TABLE I.

Showing the results of the examination of blood of 17 cholera patients during the acute stage of the disease.

Number.	Cell volume.	Hæmoglobin g. per 100 c.c.	Moisture g. per 100 g. blood.	Urea mg. per 100 c.c.	Glucose mg. per 100 c.c.	Sodium chloride mg. per 100 c.c.
1	59·6	22·3	..	..	235	..
2	44·8	..	81·6	58	172	..
3	52·4	..	77·4	125	140	..
4	48·0	16·3	76·4	45	187	..
5	54·3	24·8	73·8	..	228	..
6	36·3	15·6	80·8	..	179	..
7	69·3	26·2	73·3	55	130	..
8	51·0	19·8	77·6	..	128	298
9	39·8	..	80·4	..	103	388
10	48·5	16·3	78·2	..	124	429
11	54·2	15·8	77·5	..	101	431
12	61·4	18·8	77·5	96	90	488
13	58·5	20·5	74·9	42	243	466
14	46·1	13·8	77·8	37	126	496
15	51·2	18·1	77·4	28	123	469
16	44·3	16·7	76·0	..	124	440
17	39·5	13·0	78·6	44	152	448

TABLE II.

Showing the results of the examination of the plasma of 17 cases of cholera.

Number.	Moisture g. per 100 g.	Mg. per 100 c.c. of plasma.							
		Glucose.	Sodium chloride.	Non-protein nitrogen.	Protein nitrogen.	Fibrin nitrogen.	Globulin nitrogen.	Albumin nitrogen.	Inorganic phosphate.
1	..	274	..	..	..	..	789	..	..
2	92·7	181	..	40	1,242	..	..	..	..
3	91·2	138	..	144	1,670	87	1,111	472	5·0
4	88·6	191	..	40	1,254	..	698	..	3·9
5	..	250	..	39	1,685	66	847	772	9·6
6	..	..	..	30	1,078	52	538	488	4·9
7	85·5	148	..	38	2,139	75	917	1,147	6·3
8	91·1	136	491	145	1,229	153	605	471	6·1
9	89·1	100	525	46	1,400	105	856	439	7·2
10	92·1	126	640	31	1,252	70	939	243	4·3
11	89·1	81	628	50	1,767	66	987	714	4·2
12	89·5	76	564	88	1,741	115	..	..	17·2
13	90·7	286	596	51	1,239	126	495	618	7·2
14	89·1	130	614	46	2,045	141	910	994	5·4
15	89·9	130	637	28	1,414	114	544	756	4·1
16	86·9	135	596	101	1,948	98	1,348	502	17·3
17	88·5	161	572	40	1,506	51	592	863	5·5

Table III.

Showing the concentration of moisture, glucose and chlorides in the blood and plasma.

Number.	Moisture g. per 100 g.		Glucose mg. per 100 c.c.		Sodium chloride mg. per 100 c.c.	
	Blood.	Plasma.	Blood.	Plasma.	Blood.	Plasma.
1	..	..	235	274	..	..
2	81·6	92·7	172	181	..	..
3	77·4	91·2	140	138	..	..
4	76·4	88·6	187	191	..	..
5	73·8	..	228	250	..	..
6	80·8	..	179	..	..	..
7	73·3	85·5	130	148	..	..
8	77·6	91·1	128	136	298	491
9	80·4	89·1	103	100	388	525
10	78·2	92·1	124	126	429	640
11	77·5	89·1	101	81	431	628
12	77·5	89·5	90	76	488	564
13	74·9	90·7	243	286	466	596
14	77·8	89·1	126	130	496	614
15	77·5	89·9	123	130	469	637
16	76·0	86·9	124	135	440	596
17	78·6	88·5	152	161	448	572

TABLE IV.

Summary of the results of the examination of the blood of 17 cholera patients during the acute stage of the disease and the findings in healthy individuals.

	Cholera patients. Averages.	Cholera patients. Limits.	Normal limits.*
(A) *Blood*—			
1. Cell volume ..	50·5 per cent	36·3–69·3	36–51
2. Hæmoglobin ..	18·5 g. per 100 c.c. of blood	13·0–26·2	13–16
3. Moisture ..	77·4 ,, ,, ,,	73·3–81·6	75–82
4. Glucose	162 mg. ,, ,,	90–243	60–120
5. Sodium chloride ..	435 ,, ,, ,,	298–496	450–530
6. Urea	62 ,, ,, ,,	28–125	15–40
(B) *Plasma*—			
1. Moisture ..	89·6 g. per 100 c.c. of plasma	85·5–92·7	90–91·5
2. Glucose	159 mg. ,, ,,	76–286	60–120
3. Sodium chloride ..	586 ,, ,, ,,	491–640	560–620
4. Protein nitrogen ..	1,538 ,, ,, ,,	1,078–2,139	928–1,376
5. Non-protein nitrogen	60 ,, ,, ,,	28–145	18–30
6. Fibrin nitrogen ..	94 ,, ,, ,,	51–153	32–64
7. Globulin nitrogen ..	812 ,, ,, ,,	495–1,348	192–464
8. Albumin nitrogen ..	652 ,, ,, ,,	243–1,147	544–1,072
9. Inorganic phosphate	7·2 ,, ,, ,,	3·9–17·3	2–5

* These figures for healthy individuals are from Harrison (1937). The published figures for Indian 'normals' when available were found to correspond to the figures given.

Records in Tables I to IV show that there is a wide range of variation in the concentration of the various constituents in the blood in different patients. Such variations have been recorded (though not commented upon) by previous workers. It is not possible to correlate these variations either with the clinical condition of the patient or with the concentration of blood constituents. In this connection it must be remembered that wide variations have been recorded in healthy individuals.

The clinical condition of the 17 patients examined was approximately the same, all in the acute stage of the disease. The records of these observations, however, suggest that in the acute stage of cholera there is :—

(1) An increase in the cell volume.

(2) An increase in the hæmoglobin percentage. This increase is directly correlated with the cell volume.

(3) A decrease in moisture content of the blood and plasma but this decrease is not marked in the acute stage of the disease.

(4) An appreciable increase in the urea and non-protein nitrogen.

(5) An appreciable increase in the total plasma proteins, fibrin and globulin fractions.

There is no comparative rise in the plasma albumin so that the normal ratio of globulin/albumin is reversed in a number of patients.

(6) An increase in the inorganic phosphates.

(7) An appreciable increase in the glucose concentration in the blood and the plasma.

(8) A diminution in the concentration of the sodium chloride in the blood and in the plasma, but this diminution is not marked.

It has been felt that the data recorded above although more extensive and based on a larger series of cases than much of the data recorded in the literature is not sufficient to justify any exact determinations as to the variation occurring in the physical and chemical state of the blood in the acute stage of cholera. The literature bearing on the chemistry of cholera has been reviewed by Loh and Tai (1936) and it is unnecessary therefore to review the literature here, suffice it to say that since the investigations of Rogers (1909) and Shorten (1918) and Dhar, Dhar and Adhyee (1930) very little work has been published in India on the blood chemistry of so important a disease as cholera in which such profound disturbances in the chemical composition of the blood are known to occur. It is interesting to recall that elaborate investigations were carried out by O'Shanghnessy and others during the cholera epidemic in London of 1831-32 and later by Garrod and Parker in the epidemic of 1849. Based on these investigations, Garrod, quoted by Baly and Gull (1854), came to the following conclusions :—

(1) That in cholera, the physical characters of the blood are altered and that its tendency is to become thicker, tar-like, and less coagulable.

(2) That the proportion of water is much diminished.

(3) That the specific gravity of the serum is very high, which is due to the increase of the solid portion of the serum, and especially of the albumin; and that this fluid also tends to become less alkaline in its reaction.

(4) That with regard to the salts of the serum, some doubt exists as to their excessive diminution.

(5) That urea usually exists in increased quantities in cholera blood, but that the amount differs considerably in the different stages of the disease.

SUMMARY.

The blood from 17 cholera patients in the acute stage of the disease has been examined for the cell volume, hæmoglobin, blood and plasma moisture, blood and plasma glucose, blood and plasma chlorides (expressed as sodium chloride), blood urea, plasma non-protein nitrogen and plasma inorganic phosphates, the plasma proteins and the fractions of albumin, globulin and fibrin. The results are summarized in tabular form. The conclusions that can be deduced from the available data have been outlined.

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CHEMICAL CONSTITUENTS OF THE STOOL OF CHOLERA PATIENTS.

BY

H. GHOSH, M.B., M.S.P.E. (Paris),

Officer-in-Charge, Cholera Toxin & Immunity Inquiry, I. R. F. A.,

AND

R. K. CHAKRABORTY, M.Sc.

(An Inquiry under the Indian Research Fund Association.)

(From the Indian Institute for Medical Research, Calcutta.)

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THIS investigation was undertaken with a view to obtain positive data as to the nature and quantity of the chemical constituents such as sodium chloride, alkaline bases, carbonates, ammonia, protein, non-protein nitrogen, phosphorus, sulphur, etc. eliminated with the enormous quantity of liquid stool passed by cholera patients.

It was thought that some data might be obtained regarding the formation of cholera toxin in the intestine and the relationship between the elimination of some of the chemical constituents of the tissues and the clinical symptoms of cholera such as suppression of urine and acidosis. In the following experiments attempts were made to determine the presence and to estimate the quantity, if present, of the following chemical substances in cholera stool, viz. total solids, total alkali, total chlorides, total protein, non-protein nitrogen, ammonia nitrogen, phosphorus, sulphur, carbonates and occult blood. Stools of cases positively diagnosed by bacteriological finding as cholera was taken into consideration. The stools were collected from the Cholera Ward of the Chittaranjan Hospital, Calcutta, during the epidemic in Calcutta extending over the months of May, June, July, August and September 1938. In the Table below the specific gravity of the blood of each case on admission has been shown.

ESTIMATION OF DIFFERENT CHEMICAL CONSTITUENTS.

Methods used.

All the experiments were made with fresh samples of stools. The stools were collected in a wide-mouthed bottle containing toluol.

Total solids.—Twenty c.c. of fæces was taken in a weighed evaporating dish and dried on a water-bath; when completely dried, the basin was kept in a desiccator and weighed again.

Total alkali.—This was determined by titrating 10 c.c. of fæces with a decinormal hydrochloric acid solution using methyl orange as indicator.

Total chlorides.—Whiteborn's (1921) method was applied for the estimation of chloride. The protein was precipitated from stool by tungstic acid. The chloride was then precipitated from the stool filtrate by means of standard silver nitrate solution in presence of nitric acid and the excess of silver titrated with standard thiocyanate solution using ferric ammonium sulphate as an indicator.

Total protein.—Total protein was estimated by Kjeldahl method.

Non-protein nitrogen.—Non-protein nitrogen was determined from protein-free stool filtrate by microkjeldahl method using a sulphuric and phosphoric acid mixture for the digestion and the ammonia formed was estimated colorimetrically after direct Nesslerization of the digested mixture.

Total phosphorus.—Total phosphorus of stool was determined by Whiteborn's (1924) modification of the Bell-Doisy procedure:—

Procedure.

One c.c. of stool was taken in a hard-glass test-tube. One c.c. of concentrated sulphuric acid and 1 c.c. of concentrated nitric acid and a glass-bead were introduced into the tube. The mixture was digested over the naked flame of a micro-burner for about 10 minutes. The heating was stopped when the tube was nearly filled with fumes. It was then allowed to cool for about 3 minutes. Two c.c. of a 20 per cent solution of sodium sulphite and another glass-bead were added. The mixture was again heated as before for about 2 minutes. The heating was stopped as soon as the white fumes appeared. After cooling a minute or two, 5 c.c. of distilled water was added and the tube was cooled in cold water. In a similar tube 5 c.c. of standard phosphate solution containing 0·01 mg. of phosphorus per cubic centimetre was taken and 1 c.c. of concentrated sulphuric acid was added and cooled.

To each tube 2 c.c. of acid molybdate solution (5 per cent ammonium molybdate in 2 N sulphuric acid), 2 c.c. (20 per cent) sodium sulphite and 1 c.c. (2 per cent) of hydroquinone solution were added in the given order, shaking after each addition. The volume in each tube was then made up to 15 c.c. with water. The tubes were closed with rubber-cork and inverted twice. The tubes were kept in a boiling water-bath for 10 minutes and then cooled. The estimation of total phosphorus was made by comparing the two tubes in a colorimeter.

Total sulphur.—The method adopted was that of Benedict (1909). To 10 c.c. of stool in a porcelain evaporating dish, 5 c.c. of Benedict's total sulphur reagent was added. The mixture was evaporated to dryness on a water-bath. The evaporating dish was then heated carefully until the entire residue was blackened and then finally heated to redness for about 10 minutes after the black residue which first fused became dry. The flame was then removed and the dish allowed to cool.

Ten to twenty c.c. of dilute hydrochloric acid was added and the dish was warmed gently until the contents completely dissolved and a clear sparkling solution obtained. The solution was washed quantitatively into an Erlenmeyer flask and diluted to about 100 c.c. with distilled water. Ten c.c. of a 5 per cent solution of barium chloride was added drop by drop. At the end of an hour or later the mixture was filtered through a Gooch crucible which had previously been weighed after ignition and cooling in a desiccator. The precipitate was washed with about 200 c.c. of water. The crucible and its contents were then dried, ignited, cooled and weighed.

Ammonia.—Ten c.c. of stool was shaken with purmutit powder which removed the ammonia from the solution. The ammonia was set free from the silicate by treatment with alkali solution. This was then Nesslerized and compared with a standard ammonia solution treated in the same way.

Carbonate.—The carbonate present in stool was decomposed by the addition of hydrochloric acid and the carbon dioxide evolved therefrom was passed through a baryta solution of known strength by means of carbon-dioxide-free air aspiration. After the reaction, excess of baryta was titrated against a standard acid. Carbonate was then calculated in terms of carbon dioxide.

The presence of occult blood was detected by benzidine reaction.

TABLE.

Serial number.	Specific gravity of blood on admission.	Total solids (g.) per 100 c.c. of stool.	Total alkali in 100 c.c. of stool equivalent to N/10HCl.	NaCl per 100 c.c. of stool.	Protein (g.) content per 100 c.c. of stool.	N.P.N. (mg.) content per 100 c.c. of stool.	Total phosphorus (mg.) content per 100 c.c. of stool.	Total sulphur (mg.) content per 100 c.c. of stool.	Carbonate calculated in terms of (g.) CO_2 per 100 c.c. of stool.	Occult blood test.	Percentage of ammonia nitrogen (mg.).
1	1,062	1·55	60·0	340·3	0·58	26·7	47·6	13·4	0·125	..	..
*2	1,062	3·63	34·0	380·0	2·28	60·0	83·3	18·0	0·063	..	..
*3	1,062	5·90	62·0	500·0	2·45	37·5	166·0	9·6	0·125	..	..
4	1,066	1·78	51·0	463·0	0·83	33·3	55·5	11·2	0·103	..	..
5	1,066	2·08	34·5	490·0	1·14	50·0	40·0	3·4	0·063	..	..
6	1,060	3·20	45·0	521·0	2·19	60·0	83·3	12·6	0·089	..	..
7	1,064	1·69	50·0	428·0	0·73	42·9	33·3	14·4	0·096	..	..
*8	1,062	5·12	60·0	270·1	3·30	75·0	40·0	38·7	0·085	..	..

* These stools were collected after considerable delay.

TABLE—*concld.*

Serial number.	Specific gravity of blood on admission.	Total solids (g.) per 100 c.c. of stool.	Total alkali in 100 c.c. of stool equivalent to N/10HCl.	NaCl per 100 c.c. of stool.	Protein (g.) content per 100 c.c. of stool.	N.P.N. (mg.) content per 100 c.c. of stool.	Total phosphorus (mg.) content per 100 c.c. of stool.	Total sulphur (mg.) content per 100 c.c. of stool.	Carbonate calculated in terms of (g.) CO_2 per 100 c.c. of stool.	Occult blood test.	Percentage of ammonia nitrogen (mg.).
9	1,066	1·65	50·0	515·7	0·56	37·5	13·3	16·5	0·101	..	..
10	1,066	1·97	63·0	463·1	0·88	42·9	24·4	13·9	0·129	..	..
11	1,065	1·48	48·0	550·8	0·44	27·3	83·3	8·2	0·109	..	..
12	1,065	1·36	45·0	585·8	0·39	27·3	41·7	8·6	0·089	..	..
13	1,066	1·39	53·0	498·1	0·26	18·2	142·9	13·7	0·111	..	..
14	1,066	1·58	55·0	550·8	0·26	22·6	43·5	12·5	0·085	..	..
15	1,064	1·76	34·0	498·1	0·28	33·3	47·6	13·2	0·015	+	6·6
16	1,065	2·19	52·0	419·2	1·28	46·2	100·0	10·9	0·018	+	7·1
17	1,064	1·45	45·0	498·1	0·52	27·0	64·5	3·9	0·095	+	8·5
18	1,066	1·31	48·0	524·5	0·42	33·3	43·5	5·6	0·013	+	6·9
19	1,066	1·34	38·0	533·2	0·44	33·3	45·5	11·5	0·057	+	6·3
20	1,064	1·46	48·0	375·4	0·70	36·3	22·2	15·1	0·014	+	8·9
21	1,066	1·67	54·0	463·1	0·61	21·4	111·0	21·9	0·106	+	8·3
22	1,066	1·48	43·5	419·2	0·61	35·3	62·5	20·6	0·088	+	7·2
23	1,064	1·26	55·0	401·7	0·35	18·2	33·3	19·9	0·096	+	5·0
24	1,065	1·39	43·5	445·5	0·61	17·1	28·6	15·1	0·088	+	10·0
25	1,066	1·87	62·0	252·6	1·04	46·2	41·7	12·8	0·028	+	12·5
26	1,066	1·66	51·0	515·7	0·69	48·0	35·7	7·3	0·105	+	9·5
27	1,066	1·76	62·0	419·2	0·96	33·3	43·4	13·3	0·088	+	9·1
28	1,064	1·71	45·0	375·4	0·86	31·6	40·0	18·8	0·083	+	8·7
29	1,066	2·07	72·0	290·0	0·94	40·0	71·4	19·2	0·072	+	17·6
30	1,066	1·87	46·0	498·1	1·87	40·0	40·0	13·5	0·083	+	12·5
31	1,066	2·96	61·0	252·6	1·88	41·4	52·6	18·7	0·088	+	10·0

Thirty-one samples of cholera stool have been analysed. The stools in all cases were highly alkaline. The average alkalinity has been found to be nearly equivalent to 50·7 c.c. of N/10HCl per 100 c.c. of stool. The average percentage of sodium chloride was found to be 443·2 mg. Protein, non-protein nitrogen, ammonia, phosphorus, sulphur and carbonate contents per 100 c.c. of stool were found to be 0·95 g., 36·9 mg., 9·1 mg., 57·5 mg., 14·1 mg. and 0·081 g., respectively. The percentage of total solid material has been found to be 2·05 g. The tests for occult blood were positive in all the samples of stools examined. The specific gravity of blood of the patients varied from 1,062 to 1,066.

Conclusions.

It will be evident from the Table that all cholera stools are highly alkaline and it seems positive that alkaline reaction of the medium is distinctly favourable to the formation of cholera toxin. This is also corroborated by the fact that *Vibrio choleræ* grow abundantly in highly alkaline medium.

Elimination of alkaline base and chlorides is also considerable. This leads to acidosis and disturbs the osmotic balance. The suppression of urine may be partially due to the disturbance of this osmotic balance.

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Ind. Jour. Med. Res., **28**, 2, October, 1940.

STUDY OF THE EIJKMAN TEST AND MODIFICATIONS AS GIVEN BY *COLIFORM* ORGANISMS ISOLATED FROM HUMAN FÆCES.

BY

R. BANERJEA,

Public Health Laboratory Practice Department, School of Tropical Medicine, Calcutta,

AND

A. K. SEN,

Public Health Laboratory Practice Department, School of Tropical Medicine and Bengal Public Health Laboratory, Calcutta.

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HISTORY.

EIJKMAN TEST was first introduced by Eijkman in the year 1904 who showed that pure waters did not contain even in 300 c.c. any organism capable of fermenting glucose at 46°C. The test did not attract much attention till Leiter, in America, in 1929, tried to popularize it. In the intervening period Heheworth (1912) showed that only 38·8 per cent of his strains of *B. coli* isolated from human dejecta fermented glucose at 46°C. Of similar cultures by Barth (1930) only 37 per cent showed gas at 46°C. Leiter (*loc. cit.*), however, found that the Eijkman-fermentation test at 46°C. applied to water was selective for *B. coli*; other organisms ordinarily present in water were inhibited or destroyed. He showed that the Eijkman test was uniformly positive for *coli* isolated from fæces of human beings and warm-blooded animals.

Brown and Skinner (1930) on the other hand reported that only a small percentage of *B. coli* produced gas in Eijkman's medium even in 48 hours and that *B. ærogenes* was not completely eliminated. Taylor and Goyle (1931) found Eijkman test giving an indication of the sanitary quality of water which closely corresponded with known risk of pollution.

In view of the limitations of the Eijkman test, William, Weaver and Scherago (1933) modified the Eijkman's original medium and found that this modified medium was more reliable for the detection of fæcal *B. coli.*

Webster and Raghavachari (1934) working in Madras found the test unreliable. A water from a source exposed to pollution and showing true *coli* in very small quantities, e.g., 0·1 c.c., was found by them to give a negative test with quantities up to 50 c.c. They found that *ærogenes* sometimes survived 24 hours in Eijkman's medium at 46°C.

A committee of the Ministry of Health in England (1940) following William *et al.* advocated a modified formula for the test. A temperature of 45°C. was advocated as in the experience of the committee 46°C. temperature recommended by Eijkman was too high.

Wilson (1935) also found the temperature 45°C. to be too high and held that a temperature in the medium of 44°C. was far more suitable for growth and gas production, and he found MacConkey broth to give more satisfactory results than the modified Eijkman medium used by William *et al.* (*loc. cit.*).

Recently, Perry and Hajna (1937) reported that buffers added to Eijkman medium enhanced gas production both at 37°C. and 46°C.

The aim of the present work was to find out how far *coliform* organisms isolated from human fæces gave the positive test when cultured in Eijkman's medium as modified by William *et al.* and in the modified media recommended by others.

The *coliform* organisms were classified into fæcal, intermediate and *ærogenes* groups. The classification was based on the Voges-Proskauer, the methyl red and the citrate tests. Fæcal *coli* gave negative V. P., positive M. R. and failed to grow in the citrate medium. The intermediates behaved in the same way but they could utilize and grow in the citrate medium. The *ærogenes* and the *cloacæ* on the other hand gave a positive V. P. reaction, a negative M. R. reaction and like the intermediates grew well in the citrate medium. Each of those, the fæcal, intermediate or the *ærogenes*, could be further classified into types I and II by the indol reaction. *Cloacæ* was distinguished from the rest in being a gelatin liquefier.

V. P.− M. R.+

Citrate− | Citrate+

Fæcal *coli* | Intermediates

V. P.+ M. R.−

Citrate+

Gelatin− | Gelatin+

Ærogenes | *Cloacæ*

The true significance of the presence of the intermediates and the *ærogenes* groups of *coliform* bacteria in any sample of water was still not well understood. The fact remained that the organisms of these groups were also found in human fæces though in small numbers as would be seen from Table I, column (2).

We made no attempt in this work to study the significance of the Eijkman test as obtained with the organisms of the *ærogenes* and the intermediate groups and concentrated our attention mainly on the citrate negative groups.

Experimental.

Fæces of Indian patients not suffering from any intestinal diseases were cultured for obtaining strains of *coliform* organisms. The collection of fæces was done with all possible precautions to avoid contamination from other sources. The fæces were passed into sterilized bowls, brought over to the laboratory and at once cultured. The number of strains of *coliform* bacteria isolated and studied was 100. Out of these 87 belonged to the fæcal type, 9 to the *ærogenes* type and 3 to the intermediate type, 1 being irregular.

For doing the Eijkman test four different kinds of media were used. The formulæ of these are given below :—

(1) Single strength Eijkman (modified by William *et al.*, vide *Report of the Committee of the British Ministry of Health*)—

Peptone	5 g.
Beef extract	3 g.
Dextrose	5 g.
Water to	1,000 c.c.

(2) Double strength Eijkman (modified by William *et al.*, vide *Amer. Jour. Hyg.*, **17**, p. 432).

(3) Buffered glucose (Perry and Hajna, *Jour. Bact.*, **33**, 4, p. 339)—

Peptone	15 g.
Sod. chloride	5 g.
K_2HPO_4	4 g.
KH_2PO_4	1·5 g.
Glucose	5 g.
Water to	1,000 c.c.

(4) Double strength MacConkey broth—

Sod. taurocholate	10 g.
Lactose	20 g.
Peptone	40 g.
Sod. chloride	10 g.
Water to	1,000 c.c.

The incubator used by us was an electrically heated one of Hearson's type with a capsule to give a temperature of about 44°C. as advocated by Wilson. The temperature variation in the incubator was $\pm 0{\cdot}8$ as given by the thermometer put in the usual position of the incubator. The temperature in the actual medium was however found to be fairly constant, variation being at most $\pm 0{\cdot}2$. We always kept a rack containing a test-tube quarter filled with water and a thermometer

immersed in it. We were guided by the temperature recorded by this internal thermometer. The incubator was regulated to 46°C. instead of 44°C. when using media other than MacConkey broth. Our results using the different media with 100 *coliform* organisms isolated from human fæces were as given in Table I:—

TABLE I.

Organisms.	Number of strains.	NUMBER POSITIVE.			
		Single strength Eijkman (at 46°C.).	Double strength Eijkman (at 46°C.).	Buffered glucose (at 46°C.).	Double strength MacConkey broth (at 44°C.).
(1)	(2)	(3)	(4)	(5)	(6)
Fæcal *coli* ..	87	42	63	51	77
Ærogenes ..	9	3	2	3	4
Intermediate ..	3	0	1	2	1
Irregular ..	1	1	0	0	1

DISCUSSION.

Out of 87 citrate-negative fæcal *coliform* organisms only 42 or 48·3 per cent gave positive reaction in single strength Eijkman medium, the remainder, namely 51·7 per cent, failed to give the positive test. Buffered glucose gave somewhat better results, the percentage positive being 58·6. When double strength media were used the number giving positive reaction decidedly increased and double strength MacConkey broth gave the best results as given in Table II:—

TABLE II.

Organisms.	Number of strains.	PERCENTAGES POSITIVE.			
		Eijkman single strength.	Eijkman double strength.	MacConkey broth double strength.	Buffered glucose.
(1)	(2)	(3)	(4)	(5)	(6)
Fæcal *coli* ..	87	48·3	72·4	88·5	58·6

The first 35 strains were inoculated both into single strength and double strength MacConkey broth and the latter proved superior to the former for obtaining positive results as given in Table III :—

TABLE III.

Organisms number.	MacConkey broth single strength.		MacConkey broth double strength.	
	Positives.	Negatives.	Positives.	Negatives.
Fæcal *coli* 35 ..	25	10	33	2

CONCLUSIONS.

A large percentage, 51·7 per cent, of citrate-negative lactose-fermenting *coliforms* isolated from human fæces failed to give a positive Eijkman test as carried out with single strength Eijkman's medium. Double strength Eijkman medium seeded with the same organisms gave a larger number of positive results, 72·4 per cent, but even better was the double strength MacConkey broth incubated at 44°C., in which 88·5 per cent gave positive results.

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CAPILLARY TUBES FOR THE DISTRIBUTION OF INDIVIDUAL DOSES OF BACTERIOPHAGE.

BY

MAJOR C. L. PASRICHA, I.M.S.,

AND

M. N. LAHIRI.

(From the Cholera Bacteriological Inquiry, Indian Research Fund Association, School of Tropical Medicine, Calcutta.)

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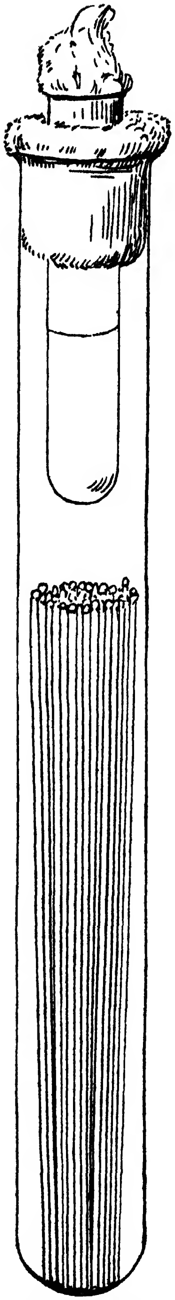
D'HERELLE (1930) in discussing the quantity of bacteriophage for therapeutic purposes concluded that the quantity used is of less importance in bacteriophage therapy than in most other therapeutic procedures. The important consideration is the virulence of the bacteriophage rather than the amount. This worker has repeatedly stressed in a number of publications that bacteriophage in the presence of susceptible organisms perpetuates itself and the amount administered does not determine the amount of bacteriophage which is ultimately developed in the body. d'Herelle and the majority of other workers who have used bacteriophage as a therapeutic agent have accepted a dose of 2 c.c. of bacteriophage as a standard individual dose. A search of the relevant literature has failed to show any cogent reasons for this particular amount and an inquiry into this subject showed that smaller amounts would suffice. A method of filling small individual doses is described below :—

Capillary tubes (about 5½″ long and 2 mm. in diameter) similar to the tubes used by certain manufacturers of smallpox vaccine lymph are used for filling



TEXT-FIGURE.

individual doses of bacteriophage. One end of the capillary tubes is sealed. About 50 such tubes can be arranged mouth downwards in an 8″ × 1″ test-tube. Bacteriophage is filtered direct into the tube by arranging a Pasteur-Chamberland L_3 candle as shown in the Text-figure. The tubes are filled by vacuum and can be readily sealed. A larger number of capillary tubes can be arranged in an 8″ × 2″ test-tube.

Each of these capillary tubes contains approximately 0·25 c.c. of bacteriophage which amount can be given conveniently in a teaspoonful of water. If desired the dose can be placed in a capsule and the capsule swallowed.

This amount of bacteriophage when swallowed is sufficient to allow the recovery of the bacteriophage from the stools.

In addition to the low cost of production another advantage in the use of capillary tubes for individual therapeutic doses of bacteriophage is the comparative ease of packing and distribution of bacteriophage.

SUMMARY.

A simple method of the distribution of individual doses of bacteriophage is described. The advantages are the ease of preparation of a large number of doses, their low cost and the ease in packing and transport.

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A 'DILUTION METHOD' FOR THE ISOLATION OF PATHOGENIC BACTERIA FROM FÆCES.

BY

MAJOR C. L. PASRICHA, I.M.S.,

G. PANJA, M.B., D.BACT.,

AND

B. M. PAUL.

(From the Department of Bacteriology and Pathology, School of Tropical Medicine, Calcutta.)

[Received for publication, May 10, 1940.]

DURING the course of certain experiments in which MacConkey's medium (bile-salt neutral-red lactose agar) was inoculated with serial dilutions of samples of fæces from a typhoid carrier it was noted that the number of colonies of *Bacterium typhosum* was greater in plates inoculated with dilutions of the stool than in plates inoculated in the usual manner. Further that with certain samples of stool, plates inoculated with the loop method gave negative results whereas plates inoculated with diluted suspensions of the stool gave positive results. Similar results were obtained in three other patients in whom *Bacterium typhosum* was found by the dilution method and only after repeated examinations by the direct plating of samples of stool. Encouraging results were obtained with cholera stools. In 23 samples of stools from cholera patients 7 samples were positive for *Vibrio choleræ* by direct plating, 13 samples were positive by the dilution method and 10 samples negative by all methods. In samples of stools from five dysentery patients similar results were obtained. As this method has given satisfactory results a short note of the technique employed is given below.

An approximate 1 in 10 dilution of the stool is prepared by taking 0·5 c.c. or 1 c.c. of the stool and suspending it in 4·5 c.c. or 9 c.c. of broth, or sterilized tap-water (reaction adjusted to pH 7·4 and coloured lightly with brom-thymol blue). From this further tenfold serial dilutions are made up to 1 in 100,000,000 or more as required, and 0·5 c.c. amounts from each of the four highest dilutions are plated

immediately after preparation on suitable medium in large Petri-dishes (5-inch diameter). The inoculum is spread by a combination of rotary and tilting movements. Inoculation with a loop charged with the diluted suspension of the stool does not give satisfactory results. A little experience with this method will demonstrate the dilutions which give satisfactory plates (with distinct colonies up to 300 or 500 in number) and the number of plates used for each specimen can be reduced.

Inoculations were made from each of the dilutions immediately after preparation and at intervals up to 2 hours. The best results were obtained in plates inoculated soon after the dilutions had been made. Plates inoculated with suspensions of stool made in different diluents such as distilled water, saline, Ringer's solution, broth and tap-water showed that the several diluents gave different results particularly if there had been delay between the preparation of the stool suspensions and the plating but not appreciably if the inoculation had been made soon after preparation of the dilutions. Distilled water and saline were definitely bactericidal probably due to the fact that the distilled water was from a copper still. Distilled water from a porcelain or all-glass still showed very much less bactericidal effect. The bactericidal effect was most marked if there had been a delay of half an hour or more between the preparation of the dilutions and the inoculation of the plates. Tap-water appeared to exert no unfavourable effect on the growth of pathogenic bacteria and when used as a diluent gave approximately the same number of colonies as were obtained with nutrient broth as the diluent. Ringer's solution prepared with the laboratory (copper still) distilled water appeared to have less bactericidal effect than distilled water or normal saline prepared from it.

If the original dilutions of the stool are prepared with small amount of stool, as for example with a loopful of the stool diluted in appropriately small quantities of fluid, the results are not as good as when half or one cubic centimetre of the stool is used for the original dilution. Several of the experiments were duplicated by different workers employing the same technique and the results of these tests were amazingly consistent. As the dilutions are made with the purpose of obtaining isolated colonies and not for any quantitative estimation, a separate pipette is not necessary for each consecutive dilution.

Two explanations for the better results obtained in the isolation of pathogenic bacteria from stools by the dilution method seem possible; either that other bacteria in the stool and other substances have an inhibitory effect on the growth of bacteria and thus diminish the number of viable pathogenic bacteria or that during the process of dilution there occurs the breaking up of the 'clumps' or colonies of the pathogenic bacteria which results in an increase of their number. Experiments designed to test the first hypothesis showed that whereas the filtrates of stool had no inhibitory effect on the growth of pathogenic bacteria isolated from the same stool the addition of even a small quantity of other bacteria (the lactose-fermenting colonies) exerted a marked inhibitory effect. This effect diminished when the mixture of the two organisms was diluted and the pathogenic bacterium was recovered in plates inoculated with the diluted mixture, whereas it was not found in plates inoculated with a loopful from the original mixture. These experiments were made with *Bact.*

typhosum, *Bact. flexneri* and *V. choleræ* and the lactose-fermenting colonies isolated from the samples of stools from which these organisms had been obtained.

The second explanation based on the assumption that the invading organisms are distributed in the stools not individually but in groups or colonies appears to be the more probable explanation. These clumps of bacteria disintegrate as the result of shaking that occurs during the process of dilution and in this way there are proportionately a larger number of the pathogenic bacteria present in high dilutions of the stool than in the undiluted specimen. During the course of this work it was often noted that in some plates inoculated with the dilutions of a stool there occurred a group of non-lactose-fermenting colonies as if there had been a local shower of these organisms.

The method of dilution of the stool is simple and if the inoculation of the medium is made soon after the preparation of the dilutions the nature of the diluent used is immaterial. The results obtained with this technique indicate that this method is of considerable value in the isolation of pathogenic bacteria.

Summary.

A procedure for the isolation of pathogenic bacteria from stools is described. It consists in the inoculation of the medium with suitable dilutions of the sample of fæces. The dilutions used are such as would give discrete colonies on the plate and are best determined by trial. This method has given better results than the usual method of inoculation.

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THE MODE OF ACTION OF 'PRONTOSIL'.

BY

K. GANAPATHI,

Lady Tata Memorial Scholar,

AND

R. SANJIVA RAO.

(From the Haffkine Institute, Parel, Bombay.)

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SOON after the announcement by Domagk (1935) of the therapeutic properties of 'Prontosil', Tréfouël (M. & Mme.), Nitti and Bovet (1935) studied the anti-streptococcal action of a series of dyes of the azobenzene group with various substituents and suggested the hypothesis that the activity of 'Prontosil' is due to the sulphanilamide produced *in vivo* by reduction. In support of this, they also showed for the first time that sulphanilamide itself is active. These observations were soon confirmed by many workers. Colebrook, Buttle and O'Meara (1936) found that the dyestuff reduced with magnesium powder had a bacteriostatic action on the multiplication of the streptococci *in vitro* comparable to that of sulphanilamide. Long and Bliss (1937*a*, *b*, *c*) showed that strong reducing agents as cystein hydrochloride and sodium formaldehyde sulphoxylate 'activated' 'Prontosil Soluble' so far as bacteriostasis *in vitro* was concerned. Definite chemical proof that such a reduction product is sulphanilamide was furnished by Fuller (1937), while Feinstone, Bliss, Ott and Long (1938) concluded after some elaborate experiments that, 'so far as the mouse is concerned, the action of "Prontosil Soluble" can be attributed to its breakdown to sulphanilamide'.

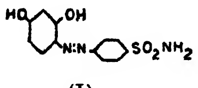
The above hypothesis of Tréfouël *et al.* has been questioned on the grounds that the amount of sulphanilamide available by reduction from therapeutic doses of the 'Prontosils' cannot account for the degree of therapeutic effectiveness observed as compared with an equal dose of sulphanilamide and that, weight for weight, the dyes are even superior to sulphanilamide in some cases (Domagk, 1936, 1937*a*, *b*; Gley and Girrard, 1936; Colebrook and Purdie, 1937; Rosenthal, Bauer and Branham, 1937; Bigler and Haralambie, 1939). In a previous communication,

one of us (Ganapathi, 1940) had pointed out another apparent objection. In view of the importance of the hypothesis of Tréfouël *et al.* in the synthesis and study of new compounds of this group, we investigated this question.

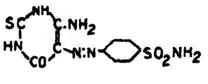
Till now, about forty dyes of this group representing a variety of structures have been tested in experimental streptococcal infections. Of these, excluding 'Prontosil Red', 'Prontosil Soluble' and 'Rubiazol', only three compounds (I), (II), and (II*a*) possess anti-streptococcal activity comparable to that of the 'Prontosils' in experimental infections in mice. The rest possess very little or no activity; however, all these should, theoretically, undergo reduction in the test-tube with strong reducing agents to yield sulphanilamide in quantities, in most cases, far greater than that obtainable from an equal weight of 'Prontosil Soluble'. If the hypothesis of Tréfouël *et al.* (1935) be correct, then, the poor activity of the above dyes should be due to one of the reasons: (*i*) they do not liberate enough sulphanilamide in the body to produce the distinct therapeutic effect, or (*ii*) the other component liberated on reduction of the dyes interferes with the activity (Holman and Duff, 1938). Since the second alternative is not plausible, we undertook to investigate whether all the active dyes liberate sufficient sulphanilamide in the blood following their administration and the inactive ones do not.

Experimental procedure and results.

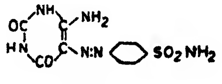
The blood concentrations of sulphanilamide following the ingestion of 'Prontosil' and 'Prontosil Soluble' have already been determined (Fuller, *loc. cit.*, Feinstone *et al.*, *loc. cit.*). Of the other three active dyes mentioned above, (I) and (II) were chosen for study since the compounds (II) and (II*a*) are very similar in properties. Among those which are very little or not active, the compounds (III)*, (IV) and (V) were chosen as the typical representatives of the different



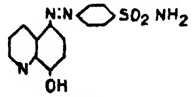
(I)



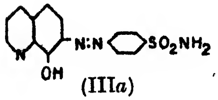
(II)



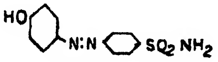
(II*a*)



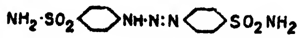
(III)



(III*a*)



(IV)



(V)

groups. These compounds were prepared by the methods described in the literature and particular care was taken to see that they were all free from even traces of sulphanilamide.

Ten milligrams each of the drugs (which represent the therapeutic doses in the usual animal experiments) suspended in ten per cent gum acacia solution were fed

* The structure (III*a*) assigned to this compound by McLeod (1938) is incorrect; the coupling in 8-hydroxy-quinoline will take place at position 5 and not at 7 (*cf.* Renshaw, Friedman and Gajewski, 1939).

to mice. After about 4 to 5 hours (when the blood concentration of sulphanilamide is known to be maximum), the heart blood was taken (about 0·5 c.c.) and the concentration of free sulphanilamide estimated colorimetrically by the method of Bratton and Marshall (1939). (The possible interference of the other component of the dyes in these estimations have been taken into account by running blanks; the effect in many cases is negligible.) The results obtained are given below in Table I:—

TABLE I.

No.	Compound fed.	Anti-streptococcal activity.	Blood concentrations of free sulphanilamide in mg. per cent.		
			I	II	III
1	'Prontosil Red' ..	Active	2·15	1·92	..
2	Compound I ..	Equal to 'Prontosil' (*a*)	2·0	1·8	..
3	Compound II ..	Slightly < sulphanilamide (*b*)	2·1	2·0	1·6
4	Compound III ..	Very slight or nil (*c*)	0·05	0·08	0·05
5	Compound IV ..	Very slight (*a*)	0·05	< 0·05	< 0·05
6	Compound V ..	Very little (*d*)	0·08	< 0·05	< 0·05

(*a*) Tréfouël *et al.* (1937).
(*b*) Ganapathi (*loc. cit.*).
(*c*) McLeod (*loc. cit.*).
(*d*) Gray, Buttle and Stephenson (1937).

In the above experiments, the blood concentrations of the dyes themselves could not be determined since the blood filtrates after the precipitation of the proteins were all colourless, the dyes being probably adsorbed by the proteins.

Since considerable amount of attention has been given to the *in vitro* activation of 'Prontosils' by reduction, some reduction experiments in the test-tube were carried out. All the six dyes, by drastic reduction (with tin or stannous chloride and hydrochloric acid), do yield sulphanilamide. But attempts were made to see whether a difference in rate of reduction between them could be found to explain the results obtained above. The three reducing agents which have been reported to 'activate' 'Prontosil' *in vitro* are magnesium powder (Colebrook *et al.*, *loc. cit.*), cystein hydrochloride (Bliss and Long, 1937) and sodium formaldehydesulphoxylate (Long and Bliss, 1937*a*, *b*, *c*). In the case of magnesium powder, the exact conditions of reduction have not been described, while cystein hydrochloride has been used to reduce 'Prontosil Solution' (2·5 per cent) in the broth itself. In the

present series of experiments, due to the very low solubility of the dyes chosen, the reduction could be attempted only in very low concentrations (1 in 20,000 or less). By adding very large excess of the last two reducing agents, appreciable amounts of sulphanilamide were formed in all cases; but by using small amounts of the reducing agents, the results obtained were surprising. A typical set of experiments is as follows :—

Twenty-five mg. of the finely powdered dyestuffs (the six mentioned above) was triturated with 50 c.c. of 0·05N hydrochloric acid and to each was added 50 mg. of magnesium powder in one set of experiments and 100 mg. of cystein hydrochloride in another set (in the latter the solutions were kept in tightly stoppered flasks to avoid atmospheric oxidation of the cystein hydrochloride). The excess of the dyes was removed from the solutions by adsorption on charcoal, after fifteen minutes in the first set and on the next day in the other set. The sulphanilamide produced in these was estimated colorimetrically, allowance being made for the possible interference of the second component produced by reduction. In the cases of 'Prontosil Red' and compound (I), no appreciable amount of sulphanilamide could be detected, while in the case of the compound (II) about 0·16 mg. to 2·5 mg. per cent of it was present. With the other dyes (III), (IV) and (V) 0·06 mg. to 0·09 mg. per cent of sulphanilamide was present when reduced with magnesium and only traces with cystein hydrochloride. It is interesting to note that Barron and Jacobs (1937) have reported that 'Prontosil Red' is not reduced by glutathione. Since these test-tube experiments do not at all correspond to the reduction processes in the animal body, we can attach no more importance to the *in vitro* reduction experiments than their having helped to shift our attention from the dyes to sulphanilamide and related compounds.

Discussion.

It is well recognized that the 'Prontosils' do undergo reduction in the animal body to yield sulphanilamide; but the question is whether, according to the hypothesis of Tréfouël *et al.*, the activity of the 'Prontosils' is due *only* to the sulphanilamide liberated by reduction. The results given in the table indicate that in the case of 'Prontosil' and the other dyes (I) and (II) which are equally active, a distinct amount (1·6 mg. to 2·1 mg. per cent) of sulphanilamide is produced in the blood following their administration, whereas in the case of the others (III), (IV) and (V) which are very little active, only traces of sulphanilamide could be detected beyond correct estimation. The blood concentration of sulphanilamide detected in the first three cases compares favourably with the results of other workers. Fuller (*loc. cit.*) has observed in a patient treated with 'Prontosil', blood concentrations of sulphanilamide of 0·93 mg., 2·2 mg. and 1·7 mg. per cent on the first, second and third day respectively. Feinstone *et al.* (*loc. cit.*) have found that in mice following single doses (parenteral administration) of 6 mg. and 20·5 mg. of 'Prontosil Soluble', the blood concentrations of sulphanilamide at the end of four hours were 1·3 mg. and 4·4 mg. per cent, respectively. Foster (1939) has recorded that after treatment of lactating women with 3·8 g. of 'Neoprontosil' for five days, the blood concentration of sulphanilamide

was 2·1 mg. per cent. Since the above concentrations of sulphanilamide are about the same as that produced by an equivalent amount of sulphanilamide itself, it is quite legitimate to conclude that the activity of the dyes is due to the sulphanilamide produced by reduction through some unknown agents which have very little in common with that in the *in vitro* methods tried. Since the concentrations of the dyes themselves in the blood could not be estimated and our test-tube reductions do not run parallel to those *in vivo*, it is not possible to ascertain whether the very low blood concentration of sulphanilamide obtained after the ingestion of the dyes with very little activity, is due to their poor absorption, slower rate of reduction or both.

The superiority of the 'Prontosils' over sulphanilamide reported in some cases, if actually found to be correct, may conceivably be due to the fact, as pointed out by Fuller (*loc. cit.*), that the dyes may act as depôts maintaining steady supplies of sulphanilamide which are more effective than sudden ingestion of large single doses. It has been strikingly demonstrated by Long, Bliss and Feinstone (1939) that the therapeutic effect of single doses of 10 mg. of sulphanilamide is far inferior to that obtained by giving the same amount in three divided doses of 3·3 mg. each in experimental infections (the two methods of treatments giving mortality rates of 80 and 50 per cent, respectively).

Till now, no rigorous argument has been advanced against the hypothesis of Tréfouël *et al.* (1935). A positive and definite case against it appeared to have been made by Rosenthal, Wooley and Bauer (1937) who reported that 'Prontosil Red' showed a specific curative action in infections due to the virus choriomeningitis while sulphanilamide and other derivatives were inactive. But, significantly enough, this has not been confirmed by Levaditi (1938), Ronse (1938) and Findlay and MacCallum (1938). Actually, sulphanilamide is more polyvalent in action than the 'Prontosils'. With a scheme of dosage calculated to produce a steady blood concentration effective in combating the infections, sulphanilamide should indeed be preferable to the 'Prontosils' in practical therapy, especially in view of the facts that it is colourless, far cheaper in cost and more polyvalent in therapeutic action.

Summary.

The arguments for and against the hypothesis of Tréfouël *et al.* that the activity of 'Prontosil' is due to the sulphanilamide produced *in vivo* by reduction, have been referred to. By feeding mice with 10 mg. each of six typical dyes of the benzenesulphonamide group and estimating the sulphanilamide concentration in blood after about four hours, it has been found that the three dyes which are quite active produced concentrations of 1·6 mg. to 2·2 mg. per cent, while those which are very little active produced only traces. The rates of reduction of these dyes observed in the test-tube with magnesium powder or cystein hydrochloride do not explain the above results. The data, so far available, do strongly support the hypothesis of Tréfouël *et al.*

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MAGNESIUM METABOLISM IN MAN.

BY

K. P. BASU, D.SC., Ph.D.,

AND

M. C. MALAKAR, M.SC.

(An Inquiry under the Indian Research Fund Association.)

(From the Biochemical Laboratory, Dacca University.)

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MAGNESIUM plays specific rôles in life processes. The work of Kruse, Orent and McCollum (1932, 1933) has shown that it is an element essential to the organism. Present knowledge with regard to the rôle of magnesium in nutrition has been summarized by Schmidt and Greenberg (1935) and recently by Duckworth (1939). Duckworth observes, however, that very little information is available regarding the magnesium requirements of man. The present investigation was undertaken to determine the magnesium requirements of adults and also to find out how these requirements are met by some typical Indian dietaries.

EXPERIMENTAL.

The experiments were conducted on three healthy adults: G. C. N. (19 years), S. N. D. (24 years), weighing 49 kilo each; and U. C. S. (30 years), weighing 48 kilo. All came from very poor village families and were accustomed to living on cereal-rich diets typical of those consumed by poor villagers. They lived with, and were kept under the strict supervision of, one of the authors. They too realized the importance of the investigations, and co-operated whole-heartedly in the work. They led a strictly disciplined life during the periods of experiment and took nothing but the weighed amounts of food daily supplied to them. The composition of the diets is indicated in the various tables. In each series of experiments the cereal and the pulse which were analysed were taken from the same stock every day and aliquots of vegetables, fish and milk taken daily were pooled for analysis. The experimental subjects were given daily carefully weighed diets which they consumed *in toto*. In certain experiments only distilled water was used for drinking and cooking purposes. When tap-water was used, the magnesium content of the water was taken into consideration. Each diet was consumed for six or nine days, the first three days being considered as a preliminary period to avoid any effect produced by the previous diet. Urine was collected quantitatively for 24-hour periods and preserved over toluene to which some chloroform solution of thymol was added.

The total volume of urine excreted each day was noted and then made up to a suitable volume for estimation. In order to be sure that the collection of urine was complete, the amount of creatinine eliminated daily was estimated and found to be uniformly constant. The fæces for three-day periods after the preliminary period were collected together since the daily dry weight of the fæces was found to vary considerably. Fæces were marked by carmine. These were preserved with a small quantity of glacial acetic acid, dried over a water-bath with frequent addition of alcohol, thoroughly powdered, weighed and preserved in a refrigerator in stoppered-bottles.

After the nine-day period on a particular diet, the effect of supplementing the diet daily with 275 c.c. of cow's milk was observed for six days, the urine being collected daily and analysed, while the fæces for three-day periods were collected together. The magnesium in the fæces, urine and foodstuffs was determined by precipitation with 8-hydroxyquinolin according to the method of Greenberg and Mackey (1932) as modified by Hummel, Stenberger, Hunscher and Macy (1936). Care was taken that no magnesium was carried down by the precipitate of calcium oxalate and that no calcium remained in solution.

Results.

The experimental results are shown in Tables I to VI :—

Table I.

Magnesium metabolism on a rice diet.

Experimental subject—S. N. D.

Diet (daily intake).	Daily intake of Mg (g.).	Daily urinary Mg output (g.).	Average daily fæcal Mg output (g.).	Total Mg output (g.).	Balance (g.).
550 g. polished rice ..					
60 g. lentil		0·113	..	..	..
200 g. vegetables ..	0·518	0·085	..	..	..
65 g. fish (*Labeo rohita*) ..		0·086	..	..	..
30 g. butter fat ..		Mean 0·095	0·278	0·373	0·145
Distilled water					
Above diet *plus* 275 c.c. cow's milk.	0·548	0·131	..	..	..
		0·113	..	..	..
		0·130	..	..	..
		Mean 0·124	0·342	0·466	0·082

This diet was taken for 12 days, 275 c.c. of milk being added during the last six days. The first three days of the experiment and the first three days on the milk diet, i.e. the 7th, 8th and 9th day of experiment, were considered to be preliminary periods and no collections were made on these days.

TABLE II.

Magnesium metabolism on a rice diet.

Experimental subject—S. N. D.

Diet (daily intake).	Daily intake of Mg (g.).	Daily urinary Mg output (g.).	Average daily fæcal Mg output (g.).	Total Mg output daily (g.).	Balance (g.).
550 g. highly polished rice; 60 g. lentil; 200 g. vegetables; 50 g. fish (*Labeo rohita*); 100 g. sugar; 30 g. mustard oil; Distilled water	0·299	0·994	..	..	..
		0·068	..	..	..
		0·066	..	..	..
		Mean 0·076	0·269	0·345	−0·046
Above diet *plus* 275 c.c. cow's milk.	0·328	0·066	..	..	..
		0·078	..	..	..
		0·066	..	..	..
		Mean 0·070	0·258	0·328	0
Above diet *plus* 275 c.c. cow's milk.	0·328	0·080	..	..	..
		0·069	..	..	..
		0·083	..	..	..
		Mean 0·077	0·263	0·340	−0·012

The diet was taken for 12 days, 275 c.c. of milk being added during the last six days. No collection was made during the first three days.

TABLE III.

Magnesium metabolism on a rice diet.

Experimental subject—G. C. N.

Diet (daily intake).	Daily intake of Mg (g.).		Daily urinary Mg output (g.).	Average daily fæcal Mg output (g.).	Total Mg output (g.).	Balance (g.).
550 g. polished rice ..						
60 g. lentil			0·0834	..	..	..
200 g. vegetables ..	0·336		0·088	..	..	..
70 g. fish (*Labeo rohita*) ..			0·154	..	..	..
30 g. mustard oil ..		Mean	0·109	0·262	0·371	−0·035
Distilled water						
			0·147	..	..	..
Above diet	0·336		0·137	..	..	..
			0·135	..	..	..
		Mean	0·139	0·266	0·386	−0·050
			0·120	..	..	..
			0·154	..	..	..
Above diet *plus* 275 c.c. cow's milk.	0·366		0·096	..	..	..
		Mean	0·123	0·290	0·413	−0·047
			0·158	..	..	..
			0·150	..	..	..
Above diet *plus* 275 c.c. cow's milk.	0·366		0·143	..	..	..
		Mean	0·150	0·278	0·428	−0·062

The diet was taken for 15 days, 275 c.c. of milk being added during the last six days. No collection on first three days.

TABLE IV.

Magnesium metabolism on a rice diet.

Experimental subject—U. C. S.

Diet (daily intake).	Daily intake of Mg (g.).	Daily urinary Mg output (g.).	Average daily fæcal Mg output (g.).	Total Mg output (g.).	Balance (g.).
600 g. rice (coarse and unpolished).					
60 g. lentil		0·091	..	..	..
200 g. vegetables ..					
70 g. fish (*Labeo rohita*) ..	0·384	0·108	..	..	..
30 g. mustard oil ..		0·103	..	..	..
50 g. sugar		Mean 0·101	0·313	0·414	–0·030
Tap-water					
		0·128	..	..	..
Above diet *plus* 275 c.c. cow's milk.	0·411	0·129	..	..	..
		0·135	..	..	..
		Mean 0·131	0·340	0·471	–0·06
		0·134	..	..	..
Above diet *plus* 275 c.c. cow's milk.	0·411	0·106	..	..	..
		0·135	..	..	..
		Mean 0·125	0·408	0·533	–0·122

The diet was taken for 12 days, 275 c.c. milk being added during the last six days.

TABLE V.

Magnesium metabolism on a whole-wheat diet.

Experimental subject—G. C. N.

Diet (daily intake).	Daily intake of Mg (g.).	Daily urinary Mg output (g.).	Average daily fæcal Mg output (g.).	Total Mg output (g.).	Balance (g.).
550 g. whole wheat ..					
70 g. horse gram ..		0·092	..	..	..
180 g. vegetables ..	0·617	0·123	..	..	..
30 g. butter fat ..		0·106	..	..	..
60 g. sugar		Mean 0·107	0·490	0·597	+0·020
Tap-water					
		0·103	..	..	..
Above diet	0·617	0·097	..	..	..
		0·104	..	..	..
		Mean 0·102	0·475	0·577	+0·040
		0·117	..	..	..
Above diet *plus* 275 c.c. cow's milk.	0·647	0·115	..	..	..
		0·103	..	..	..
		Mean 0·117	0·483	0·600	+0·047
		0·121	..	..	..
Above diet *plus* 275 c.c. cow's milk.	0·647	0·124	..	..	..
		0·119	..	..	..
		Mean 0·122	0·445	0·567	+0·080

The diet was taken for 15 days, 275 c.c. milk being added daily during the last six days.

TABLE VI.

Magnesium metabolism on a whole-wheat diet.

Experimental subject—U. C. S.

Diet (daily intake).	Daily intake of Mg (g.).	Daily urinary Mg output (g.).		Average daily fæcal Mg output (g.).	Total Mg output (g.).	Balance (g.).
550 g. whole wheat .. 70 g. horse gram .. 180 g. vegetables .. 30 g. butter fat .. 50 g. sugar .. .. Tap-water	0·585		0·124	..	..	..
			0·116	..	..	..
			0·112	..	..	..
		Mean	0·117	0·220	0·337	0·248
			0·114	..	..	..
			0·115	..	..	..
			0·118	..	..	..
		Mean	0·116	0·238	0·354	0·231
Above diet *plus* 275 c.c. cow's milk.	0·614		0·118	..	..	..
			0·121			
			0·128	..	..	..
		Mean	0·123	0·315	0·438	0·176
Above diet *plus* 275 c.c. cow's milk.	0·614		0·117	..	..	..
			0·133	..	..	..
			0·129	..	..	..
		Mean	0·126	0·294	0·420	0·194

The diet was taken for 15 days, 275 c.c. milk being added daily during the last six days.

THE MAINTENANCE REQUIREMENT OF MAGNESIUM FOR ADULTS.

Leitch's (1937) graphical method for determining the maintenance requirement of an element is associated with certain disadvantages and has been criticized

by Mitchell (1938). We have adopted here the procedure of Sherman in which output of magnesium near the balance point is taken to be the requirement.

The output of magnesium when the subjects were nearly in magnesium balance is indicated in Table VII :—

TABLE VII.

Magnesium output and requirement.

Experimental subject.	Diet.	Magnesium output near equilibrium (g.).	Mean Mg requirement (g.).	Reference to data.
S. N. D. (49 kilo) ..	Rice	0·373	0·370	Table I.
		0·466		do.
	Highly polished rice	0·345		Table II.
		0·328		do.
		0·340		do.
G. C. N. (49 kilo) ..	Rice	0·371	0·492	Table III.
		0·386		do.
		0·413		do.
		0·428		do.
	Wheat ..	0·597		Table V.
		0·577		do.
		0·600		do.
		0·567		do.
U. C. S. (48 kilo) ..	Rice	0·414	0·424	Table IV.
		0·471		do.
		0·533		do.
	Wheat ..	0·337		Table VI.
		0·354		do.
		0·438		do.
		0·420		do.

The mean magnesium requirement of an adult is calculated to be 0·429 g. per day, this value being based on the average of 20 metabolism experiments on three individuals. Although some meagre information on the magnesium requirements of pregnant women (Coons and Coons, 1935) and of pre-school children (Daniels and Everson, 1936) is available, very little is known regarding the magnesium requirements of healthy adults. Brull (1936) estimated that the daily requirement of magnesium for maintenance in adults was about 0·20 g. Schmidt and Greenberg (*loc. cit.*) state in their review that the requirement is 0·60 g. daily. Tibbets and Aub (1937) found that a positive magnesium balance was maintained by hospital patients on an intake of 0·220 g. per day and that medical students pursuing their normal activities were in positive magnesium balance on an intake of 0·300 g. per day. Our experimental subjects required 0·370 g. to 0·492 g. of magnesium per day for balance. When this paper was under preparation, McCance and Widdowson (1939) published a paper describing the fate of magnesium injected intravenously into persons already in magnesium balance. They found that their experimental subjects, six in number, required on an average about 0·279 g. magnesium per day for balance. In view of the relatively small amounts of magnesium present in the body it is very probable that the magnesium requirement is not proportional to the body-weight. The requirement has therefore been stated in terms of an adult.

The high magnesium requirement, which is almost of the same order as the calcium requirement, is somewhat remarkable in view of the difference in the amounts of calcium and magnesium in the body. The cereal diets of Indians who cannot generally afford to include milk in their dietaries are deficient in calcium, and investigations in this laboratory (Basu, Basak and Rai Sircar, 1939) show that they may be in negative calcium balance. This is likely to cause some degree of osteomalacia in adults, and McCrudden (1909-10) and Euler and Rydbom (1935) have shown that the percentage of magnesium in bone is very high in rickets and in osteomalacia. This circumstance might cause a greater elimination of magnesium in such persons. This investigation also shows that freely chosen dietaries may not always supply the required amount of magnesium.

Channels of excretion.

At the levels of intake used in this investigation about one-fourth of the magnesium was excreted in the urine and three-fourths in the fæces. Tibbets and Aub (*loc. cit.*) observed that about one-third of the magnesium was excreted through the kidney. McCance and Widdowson (*loc. cit.*) recorded the values 0·70 and 0·46 for the ratio urinary Mg $\div$ fæcal Mg for low and high intakes of magnesium respectively. They also found that intravenous injection of magnesium, as also of calcium into normal persons, resulted in a rapid increase in the excretion of the element by the kidney and in most cases the additional output was equal to the amount injected.

Nelson (1916-17, 1920) reported that more calcium than magnesium was usually excreted in human urine. In this investigation, however, in the case of G. C. N.

and S. N. D., the amount of magnesium excreted in the urine was 2 to 4 times the amount of calcium. The urinary excretion of calcium and magnesium was almost equal in the case of U. C. S.

Effect of milk supplement.

The addition of 275 c.c. milk, which supplied about 30 mg. of magnesium, did not improve the retention of magnesium. In fact continued intake of milk very often caused a slightly greater elimination. Somewhat similar results were obtained by Heller and Haddad (1936) who observed that a high calcium intake reduced magnesium retention and even caused loss of magnesium from the body.

Conclusion.

Sherman's (1937) contention that 'an ordinary mixed diet, unless it consists too largely of highly refined food materials, will usually furnish a safe surplus of magnesium' may not always hold good. In our investigations the experimental subjects consuming typical Indian diets were either just in equilibrium or in negative magnesium balance and the diets did not contain a sufficient margin of this element. Whole-wheat diets were superior to rice diets in this respect. For an adequate supply of magnesium Indian diets require to be supplemented with sufficient amounts of green vegetables and legumes. Milk and eggs, which are generally recommended for improving Indian diets, do not contain much magnesium.

Summary.

1. Twenty magnesium metabolism experiments were performed on three adults living on typical Indian cereal diets. The effect of supplementing the diets with milk has also been studied.

2. The maintenance requirement of magnesium calculated from those experiments in which the experimental subjects were nearly in balance appears to be 0·429 g. for an adult per day.

3. The addition of milk to the diet did not raise magnesium retention. Continual intake sometimes caused a slightly greater elimination.

4. Indian dietaries require to be liberally supplemented with vegetables and legumes in order that a sufficient amount of magnesium may be provided. Whole-wheat diets appear to be superior to rice diets in their effect on magnesium metabolism.

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STUDIES ON BASAL METABOLISM IN BOMBAY.

Part II.

BASAL METABOLISM OF BOYS.

BY

S. P. NIYOGI,

V. N. PATWARDHAN,

P. L. POWAR,

AND

M. V. SIRSAT.

(An Inquiry under the Indian Research Fund Association.)

(From the Department of Physiology, Seth Gordhandas Sunderdas Medical College, Parel, Bombay.)

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THE investigations carried out in different parts of India have now proved beyond doubt that the basal metabolic rate (B. M. R.) of adult Indians is lower than that of the Europeans and Americans. A recent publication by Sokhey and Malandkar (1939) summarizes the previous work besides confirming the statement made above. The information about the B. M. R. of Indian children is however very meagre. Recently, Wilson and Roy (1938) have reported a study of the B. M. R. of 62 boys of ages 6 to 16 years belonging to the poorer classes in Calcutta. Their results showed that the B. M. R. was lower than the DuBois standards by 9·7 to 19·5 per cent. The values, however, compared favourably with Benedict standards. The present communication contains the results of an investigation of 35 boys in Bombay between the ages of 11 and 16 years.

EXPERIMENTAL.

The subjects selected were the inmates of the David Sassoon Industrial School, Matunga, Bombay. They belonged to different communities but lived under

identical conditions. Their diet was found to be adequate in total proteins, fats and calories although deficient in animal proteins. They were examined a few days before the test to eliminate those that revealed any abnormal clinical condition. The Sanborn Metabolism Tester was used for recording the B. M. R. The technique of the test and the precautions taken to minimize errors have been fully described in the previous communication (Niyogi, Patwardhan and Mordecai, 1939).

In Table I are given the maximum, minimum and average values of several physical and physiological measurements. The results of the B. M. R. tests (3rd series) are set out in Table II.

TABLE I.

The results of basal metabolism tests with other relevant data.

A. *Age in years and months.*	
Maximum	16/1
Minimum	10/6
Average	13/7·5
B. *Weight (without clothes), kg.*	
Maximum	44·9
Minimum	27·5
Average	35·8
C. *Standing height in cm.*	
Maximum	161·9
Minimum	140·9
Average	149·6
D. *Sitting height in cm.*	
Maximum	86·4
Minimum	70·1
Average	76·8
E. *Pirquet index or Pelidisi.*	
Maximum	104
Minimum	85
Average	92·1
F. *Respiration rate.*	
Per minute during experiment	18
G. *Pulse rate per minute.*	
Average before the test	65
Average during the test	64

H. *Blood pressure (basal) in mm. Hg.*		
Average systolic		92
Average diastolic		52
I. *Vital capacity in c.c.*		
Maximum		2500
Minimum		1100
Average		1750
J. *Barometric pressure in mm. Hg.*		
Maximum		769
Minimum		756
Average		762
K. *Room temperature in °F.*		
Maximum	Dry bulb	87
Minimum	Dry bulb	76
Average	Dry bulb	81
Maximum	Wet bulb	84
Minimum	Wet bulb	61
Average	Wet bulb	75
L. *Percentage of humidity.*		
Maximum		96
Minimum		32
Average		63

The data presented in Table II have been further analysed and grouped according to age periods. The age interval for each group has been arbitrarily fixed, i.e. the boys between 10 years and 6 months to 11 years and 5 months were included in the age group 11 years and so on throughout the groups. Mean figures are given in Table III. The deviation of the values obtained by calculation from the accepted American standards (DuBois, 1936) is shown in the Graph.

TABLE II.

Basal metabolism of normal boys in Bombay. Series III.

Subject number.	Age.	Body-weight, kg. (without clothes).	Height, standing, cm.	O_2 consumption per minute, c.c.	Heat production in calories. For 24 hours. Total.	Heat production in calories. For 24 hours. Per kg.	Heat production in calories. For 1 hour. Per sq. m.	Percentage deviation from standards. Mayo Clinic.	Percentage deviation from standards. Harris and Benedict.	Percentage deviation from standards. DuBois.
81	10/6	34·3	151·7	158	1,098	32·0	37·1	−22·9	−10·9	−24·3
82	11/8	30·7	143·0	157	1,091	35·5	40·6	−14·7	−3·0	−15·4
77	12/2	34·8	146·8	141	979	28·1	33·8	−27·7	−19·3	−27·3
90	12/2	36·9	147·9	203	1,410	38·2	47·4	−0·0	+13·5	−0·0
89	12/3	31·5	145·7	173	1,202	38·2	43·2	−0·9	+3·7	−0·8
86	12/4	41·2	159·4	176	1,223	29·7	37·2	−21·1	−11·6	−20·2
79	12/6	32·0	142·3	135	938	29·3	34·6	−20·6	−17·5	−27·0
87	12/6	35·5	148·2	155	1,077	30·3	36·2	−22·1	−10·2	−23·8
83	12/8	33·4	144·5	153	1,063	31·8	37·9	−19·5	−7·5	−19·9
85	12/8	33·4	144·2	166	1,153	34·5	41·1	−12·2	−0·0	−13·1
76	13	30·6	143·0	130	903	29·5	33·6	−29·0	−17·9	−27·7
101	13	31·8	146·7	156	1,084	34·1	38·6	−17·0	−4·4	−18·0

TABLE II—*concld.*

Subject number.	Age.	Body-weight, kg. (without clothes).	Height, standing, cm.	O_2 consumption per minute, c.c.	Heat production in calories. For 24 hours. Total.	Heat production in calories. For 24 hours. Per kg.	Heat production in calories. For 1 hour. Per sq. m.	Percentage deviation from standards. Mayo Clinic.	Percentage deviation from standards. Harris and Benedict.	Percentage deviation from standards. DuBois.
111	13	30·3	142·2	154	1,070	35·3	40·9	−14·4	−1·1	−14·0
84	13/1	28·6	141·2	127	882	30·8	34·4	−28·2	−17·5	−26·8
109	13/4	35·0	148·2	167	1,160	33·1	39·6	−15·2	−1·8	−15·4
80	13/5	44·7	153·5	188	1,306	29·2	40·0	−15·7	−1·8	−14·3
102	13/5	33·0	147·0	161	1,119	33·9	39·2	−15·7	−2·7	−16·1
106	13/6	34·0	145·2	178	1,237	36·4	43·6	−7·4	+6·9	−5·8
93	13/9	41·5	156·2	164	1,139	27·4	35·2	−25·1	−13·3	−24·0
94	13/9	27·8	141·3	143	993	35·7	39·4	−16·9	−5·3	−14·3
100	13/9	36·7	155·3	185	1,285	35·0	41·9	−10·6	+3·7	−8·9
115	13/9	36·1	153·7	181	1,258	34·8	41·6	−10·4	+3·3	−10·3
108	13/10	39·0	147·6	168	1,163	29·9	38·3	−18·0	−6·0	−17·5

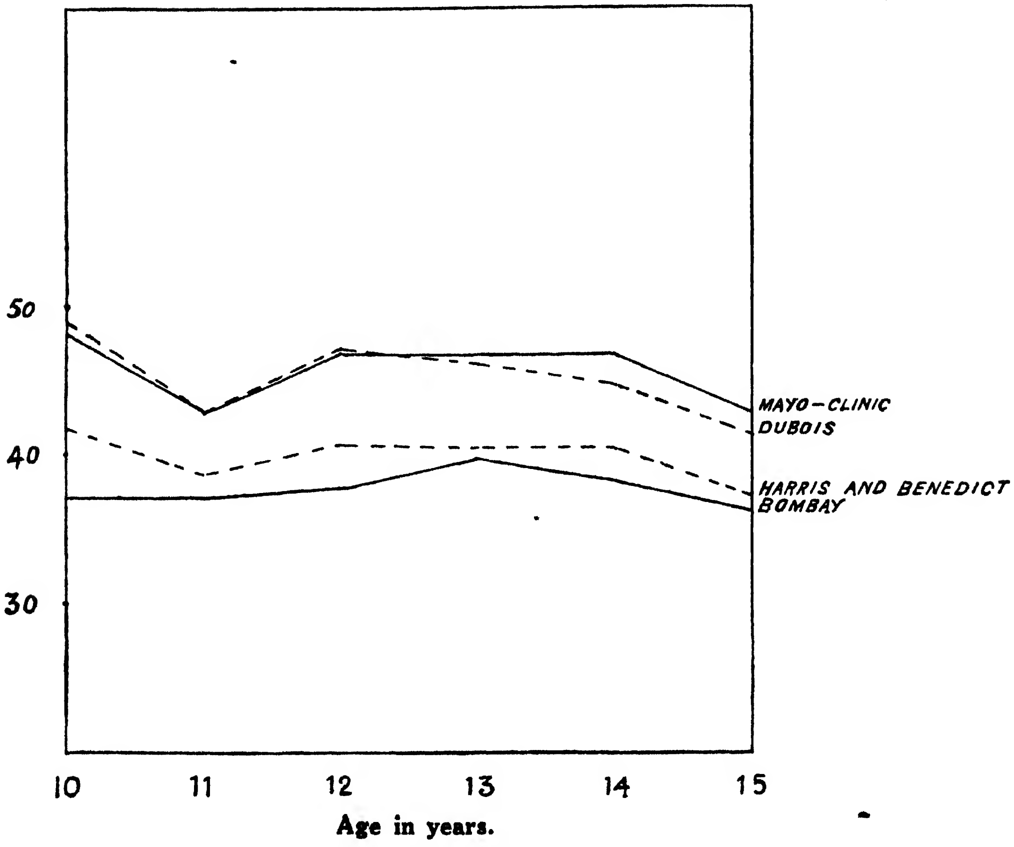
107	13/11	37·0	147·9	183	1,271	34·3	42·7	—9·4	+4·7	—7·8
105	14	31·1	149·5	151	1,049	33·7	37·4	—20·1	—7·8	—19·0
121	14/9	40·3	156·2	173	1,202	29·8	37·4	—19·9	—7·7	—18·0
124	14/9	43·1	151·8	183	1,271	30·2	39·3	—16·0	—3·5	—13·8
95	15	38·8	153·6	174	1,209	31·2	38·8	—18·3	—4·5	—14·3
99	15	44·9	161·9	195	1,355	30·2	39·2	—15·2	—2·6	—13·5
126	15/1	38·6	150·5	172	1,195	31·0	39·2	—16·5	—3·5	—13·5
118	15/3	38·0	145·7	149	1,035	27·2	34·5	—26·2	—14·8	—23·7
122	15/4	42·5	154·3	183	1,271	29·9	39·0	—16·8	—3·5	—13·5
104	15/8	36·3	154·9	169	1,174	32·3	38·5	—17·2	—5·0	—14·3
116	15/9	38·5	160·9	183	1,271	33·0	39·6	—14·9	—1·4	—11·8
110	16/1	38·0	158·8	178	1,237	32·5	39·9	—14·8	—3·2	—12·8
					Average deviations		.. ..	—16·9	—5·5	—15·8
				Series II	,,	,,	.. ..	—16·7	—4·5	—16·1

TABLE III.

Average heat production in the various age groups.

Age group years.	Number of boys.	Calories per kg. per 24 hours.	Calories per sq. m. per hour.
11	1	32·0	37·1
12	5	35·4	37·1
13	11	32·0	37·8
14	8	33·4	40·0
15	7	29·9	38·2
16	3	32·6	36·0

GRAPH.



The comparison of B. M. R. of Bombay boys with the predicted values from some American standards.

That the B. M. R. of Indian boys would be lower than that of the American and European boys was to be expected. The causes underlying this lowered metabolism may be the same as those responsible for the relatively low B. M. R. of adult Indians. Wilson and Roy (*loc. cit.*), commenting on their own results, suggest undernutrition or malnutrition as a possible factor in producing the low B. M. R. observed in their subjects. A comparison of our data with their published figures shows that the boys in Bombay were taller and heavier on an average than their subjects and yet the B. M. R. of the Bombay boys was lower than that of boys of the corresponding age groups in Calcutta. The diet of the Industrial School boy was adequate in total proteins, fats and calories although deficient in animal proteins. It is doubtful whether this deficiency of animal proteins can account for the low B. M. R. in view of the fact that our experimental results, to be published later, have shown that the B. M. R. is not appreciably influenced by an increased consumption of animal proteins, and it is not necessarily low in those whose consumption of animal proteins is low. It appears, therefore, that the low B. M. R. recorded in these tests could not be possibly due to undernutrition. The number of boys in each age group, however, is too small to permit the establishment of general averages. The consideration of the influence of puberty and the changes in B. M. R. during adolescence will, therefore, have to wait till many more observations have been made.

Summary.

The basal metabolism of 35 boys between the ages of 11 to 16 years has been determined. The average B. M. R. was lower than the Mayo Clinic and DuBois standards by 16·8 per cent and 15·9 per cent respectively and 5·0 per cent lower than the Harris and Benedict standards.

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The authors are grateful to the authorities of the David Sassoon Industrial School, Matunga, for permission to work there. They have also much pleasure in thanking Dr. Jivraj N. Mehta, the Dean, Seth G. S. Medical College, for his interest and encouragement.

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Ind. Jour. Med. Res., 28, 2, October, 1940.

STUDIES IN CALCIUM AND PHOSPHORUS METABOLISM.

Part III.

THE CALCIUM CONTENT OF SOFT TISSUES OF ALBINO RATS IN RICKETS AND HYPERVITAMINOSIS D.

BY

V. N. PATWARDHAN,

AND

R. G. CHITRE.

(An Inquiry under the Indian Research Fund Association.)

(Department of Physiology, Seth Gordhandas Sunderdas Medical College, Parel, Bombay.)

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In an earlier communication (Patwardhan and Chitre, 1938) the observations reported by other workers that there are variations in the calcium and phosphorus content of the soft tissues of normal albino rats have been confirmed and the factors responsible for these variations examined. It was shown that the diet of the animals influenced the Ca and P content of the soft tissues, since the difference between the maximum and minimum values was found to be the least among the tissues of rats belonging to the same diet group.

The information about the Ca and P content of the soft tissues in diseases characterized by a disturbance in Ca metabolism is, however, meagre and contradictory. The observations of Haury (1930) that the Ca content of muscle in the albino rat is decreased in rickets has not been confirmed by Burns (1933). Hess, Gross, Weinstock and Berliner (1932) have found that in rachitic rats the amounts of Ca and P were normal in the kidneys, lungs, skin and other soft tissues while the Ca content of brain was decidedly lower in rickets of high Ca, low P, and low Ca, high P, types. These observations of Hess *et al.* (*loc. cit.*) require confirmation, for the variations in the Ca and P content of tissues of normal rats recorded by these authors are themselves so great that any changes observed in rickets are thereby rendered difficult of interpretation (Schmidt and Greenberg, 1935).

Experiments were therefore undertaken to investigate the possible effects of a deficiency of vitamin D, and also its excess, on the Ca and P content of soft tissues.

EXPERIMENTAL.

Analytical methods.—The removal of the soft tissues, their subsequent treatment, and the methods of estimating Ca and P were the same as those described by the authors in a previous communication (Patwardhan and Chitre, *loc. cit.*).

Diets.—In Table I is given the composition of various diets referred to in the course of this paper :—

TABLE I.

Composition of diets.

Article.	Diet I.	Diet II.
A. Stock diets.		
Wheat flour	60 g.	60 g.
Gram flour	20 „	20 „
*Ankoria Baby Food	5 „	5 „
Sesame oil	5 „	5 „
Meat paste	2 „	2 „
Sodium chloride	0·5 „	0·5 „
Calcium carbonate	0·5 „	1·5 „
Disodium hydrogen phosphate ..	*Nil.*	3·6 „
Fresh whole milk	60 c.c.	60 c.c.
Ascorbic acid	6 mg.	6 mg.
N per cent	2,730 mg.	2,730 mg.
Ca per cent	390 „	789 „
P per cent	315 „	625 „
Ca/P	1·23	1·26

*Five cases of spoilt Ankoria Baby Food (a milk product) were handed over by the manufacturers to the Honorary Secretary, Bombay Presidency Baby & Health Week Association. Since the material was unfit for human consumption it has been utilized for feeding rats for the last four years. On analysis it was found to contain protein 16·6 per cent, fats 13·4 per cent, carbohydrates 65 per cent, Ca 0·13 per cent, and P 0·345 per cent. There was no particular reason to include it in the diet.

TABLE I—*concld.*

Article.			Quantity.
B. Rachitogenic diet.			
Yellow maize	..	..	75 g.
Dried meat powder	..	..	10 „
Gelatin ..	..	..	10 „
Sodium chloride	..	..	1·3 „
Calcium carbonate	..	..	3·7 „
Ca per cent	..	..	1,812 mg.
P per cent	..	..	302 „
Ca/P ..	..	..	6

The various ingredients of the diets, with the exception of milk, were mixed with an adequate amount of water and cooked. The cooked food was dried in a current of hot air at 40°C. to 45°C. and powdered. It was stored in corked bottles. A fresh stock was prepared once a week. Fresh whole milk was given to rats separately.

CA CONTENT OF SOFT TISSUES OF RACHITIC RATS.

Fifteen young rats of both sexes four weeks old were divided into three groups: (1) Seven rats were kept on the rachitogenic diet, (2) four rats were given the same diet supplemented by 60 I. U. of vitamin D as a daily supplement, and (3) the remaining four were kept on the stock diet II. The rats on the rachitogenic diet developed rickets, as was shown by bone analyses, within four to five weeks. They were killed and their tissues removed for analysis. The rats from control groups II and III were also killed at corresponding periods. The results of analyses are shown in Table II.

A statistical examination of the data shows that in only three tissues of the eight examined were there any significant alterations as a result of the deficiency of vitamin D. In the brains of rachitic rats the mean value for Ca was found to be 32·65 mg. per 100 g. of dry tissue and 44·31 mg. in the group protected by vitamin D, while in stock diet control group it was 50·78 mg. per cent. The difference between the last two values was not significant, while that between the first two values was significant. This result permits the conclusion being drawn that the Ca content of the brain was reduced as a result of deficiency of vitamin D, a finding which confirms the observations of Hess and co-workers (*loc. cit.*). In the lungs of rachitic rats, on the other hand, a significant increase was observed in the amount of Ca, the mean value being 79·50 mg. per 100 g. of dried tissue, whereas the corresponding value for lungs of rats from groups II and III were 54·22 mg. and 51·34 mg., per cent respectively.

TABLE II.

The calcium content of the soft tissues of rachitic rats.

Mg. Ca per 100 g. of dried tissue.

		Muscle.	Brain.	Spleen.	Testes.	Liver.	Heart.	Lungs.	Kidneys.
Group I 'Rickets'	Maximum	77·09	39·94	145·50	117·10	41·50	119·40	127·00	81·66
	Minimum	35·50	19·10	79·80	76·58	21·69	32·33	58·43	34·07
	Mean	60·37	32·65	115·79	96·80	29·25	75·85	79·50	53·91
	σ	14·09	7·04	33·36	..	8·59	33·32	16·94	23·72
	Standard error	5·33	2·66	12·61	..	3·25	12·59	6·40	8·95
Group II Protected from rickets by 60 I. U. vitamin D per rat per day.	Maximum	70·31	54·76	142·50	..	63·10	60·96	69·18	86·83
	Minimum	47·32	36·93	47·36	..	35·40	50·00	41·50	47·17
	Mean	58·50	44·39	98·40	62·58	48·06	54·13	54·22	63·18
	σ	11·94	7·48	44·90	..	11·20	5·27	11·32	16·83
	Standard error	5·97	3·74	22·40	..	5·60	2·63	5·66	8·41
Group III Stock (control) ..	Maximum	33·50	59·27	48·25	44·16	26·18	51·29	53·90	54·58
	Minimum	31·01	42·27	37·33	37·46	20·42	39·66	47·71	43·85
	Mean	32·35	50·79	42·55	40·81	23·92	45·57	51·34	48·67
	σ	0·23	6·84	7·17	..	8·24	5·11	2·89	5·41
	Standard error	0·12	3·42	3·58	..	4·12	2·55	1·44	2·70

The Ca content of spleen, muscle, heart and kidney show no significant differences between groups I and II. When the comparison was made between group II and group III (stock diet controls), however, significant differences in the Ca content of some tissues were observed, while in others no such changes were noticeable. It must be stressed here that rickets in rats is produced not merely by withholding vitamin D from the diet but by simultaneous gross alterations in the quantities of Ca and P in the rachitogenic diets. The change from the stock to the rachitogenic diet may, even when the diet is supplemented with vitamin D, cause certain as yet unknown variations in metabolism which might ultimately be reflected in the composition of the tissues. That such changes probably took place can be seen by comparing the Ca content of muscle, spleen, liver and heart of rats from groups II and III.

The observed alterations in the Ca content of soft tissues in experimental rickets in rats can be ascribed to vitamin D deficiency alone if the differences between the mean values of Ca in the soft tissues of rats on rachitogenic diet (group I) and of those on the same diet supplemented with vitamin D (group II) are found to be statistically significant. Significant differences between rachitic and stock diet (group III) groups alone cannot justify the drawing of similar conclusions. The Ca content of the liver of rats from these experimental groups illustrates this point. As a result of transfer from the stock diet II to the rachitogenic diet supplemented with vitamin D the Ca content of liver increased significantly. The livers of rats receiving no supplement of vitamin D to their rachitogenic diet gave values for Ca significantly lower than those from group II yet higher than those in group III (*see* Table II), with the result that the comparison between rachitic and stock diet groups shows differences which are not significant. On the strength of the difference in the Ca content of livers of rachitic rats and those receiving vitamin D supplement, however, the conclusion is justified that vitamin D deficiency caused a decrease in liver Ca. The application of similar criteria justifies the afore-mentioned conclusion that the Ca content of spleen, muscle, heart, and kidney remains unaltered in vitamin D deficiency.

The calcium content of the soft tissues of rats in induced hypervitaminosis D.

In these experiments two groups of adult rats were used, one bred and grown on stock diet I (Ca = 390 mg. per cent, P = 315 mg. per cent) and another on stock diet II (Ca = 789 mg. per cent, P = 625 mg. per cent); the only difference in the composition of these diets was in Ca and P content. Hypervitaminosis was induced by daily injections of 4,500 I. U. and 7,200 I. U. of vitamin D per rat per day for rats of groups I and II, respectively. As the result of previous experience the loss of weight and a considerably decreased food intake were considered as signs diagnostic of hypervitaminosis. The rats were killed when their food intake had fallen to 30 to 40 per cent of the original. Their soft tissues were then analysed by the methods already described. The results are given in Table III :—

TABLE III.

Ca content of soft tissues of albino rats in hypervitaminosis D.

Mg. Ca per 100 g. dried tissue.

		Muscle.	Brain.	Spleen.	Testes.	Liver.	Heart.	Lungs.	Kidneys.
Diet I (stock) (6 rats) ..	Maximum	39·80	92·30	65·01	41·88	38·70	61·70	145·60	62·50
	Minimum	29·30	65·30	36·92	38·58	21·10	51·50	97·10	47·60
	Mean	35·87	79·59	55·74	40·09	28·83	53·91	121·50	53·07
	σ	3·64	9·77	10·02	2·40	7·75	3·85	19·36	5·18
	Standard error	1·49	4·01	4·09	1·20	3·09	1·58	7·92	2·12
Rats receiving 4,500 I. U. per rat per day (10 rats)	Maximum	108·10	207·00	232·30	108·20	70·10	200·40	244·30	146·90
	Minimum	52·09	78·70	60·39	55·48	33·01	64·86	66·80	45·29
	Mean	72·60	117·18	106·58	75·86	50·23	127·08	117·99	71·11
	σ	16·26	42·65	49·90	18·25	8·77	47·55	45·11	28·09
	Standard error	5·14	13·49	15·78	5·76	2·78	15·04	14·27	8·89

Diet II (stock) (3 rats) ..	Maximum	36·10	93·70	65·10	54·70	35·80	86·39	84·55	66·07
	Minimum	34·25	60·63	38·11	40·80	20·37	37·76	53·60	42·81
	Mean	35·24	74·15	45·56	47·70	29·73	54·17	70·35	57·12
	σ	1·02	13·9	16·33	..	6·42	7·03	4·57	9·76
	Standard error	0·51	6·9	8·16	..	3·21	3·51	2·28	4·85
Rats receiving 7,200 I. U. per rat per day (8 rats)	Maximum	140·10	131·90	103·00	77·03	68·73	221·30	113·79	154·20
	Minimum	48·19	85·80	42·92	47·15	28·08	67·08	57·82	46·20
	Mean	68·49	99·98	74·08	61·99	44·92	103·83	87·88	74·30
	σ	32·43	21·04	21·11	..	14·80	38·90	24·35	36·30
	Standard error	11·74	7·43	7·44	..	5·23	13·75	3·61	12·72

Table III shows that excessive dosage of vitamin D raises the total Ca content of certain tissues. The rise was significant in muscle, brain, spleen, testes, liver and heart in both the experiments, whereas the Ca content of lung and kidney did not show a significant rise in either of the experiments. A histological examination of the tissues of these rats, however, did not reveal signs of calcification except in the kidneys of a few rats suffering from hypervitaminosis. Thus, it would appear that calcification of soft tissues probably does not depend only on the increased Ca content of the blood and the tissues but that some other factor or factors may also be necessary for the deposition of this element.

The observed changes in the Ca content of the tissues in rickets and hypervitaminosis D are, however, difficult to interpret. Hess *et al.* (*loc. cit.*) who recorded a decrease in the Ca content of the brain found that this decrease depended neither on the alkalinity of the diet, nor its Ca content, nor on the Ca content of serum; even parathyroidectomy did not affect the Ca content of the brain. The alterations observed in the present series of experiments do not show a uniform tendency, nor is it possible to explain these in the light of the known functions of Ca in the animal body. Until further information is available about the state of Ca, and its functions, in different tissues, it will not be possible to interpret data of this kind.

Summary.

1. The effects of a deficiency of vitamin D and also its excess on the Ca and P content of the soft tissues of albino rats have been studied.

2. A significant reduction in the Ca content of brain and liver of rachitic rats has been observed; the Ca content of lung was found to be increased. The Ca content of other tissues examined remained unaltered.

3. Excessive dosage with vitamin D led to an increase in the Ca content of muscle, brain, spleen, testes, liver and heart; the Ca content of lungs and kidney, however, remained unchanged. Histological examination of tissues of these rats did not reveal signs of calcification except in the kidneys of a few rats. Hence it is suggested that the calcification of soft tissues probably does not depend only on the increased Ca content of blood and tissues but some other factor or factors might also be necessary for the deposition of this element.

The authors have great pleasure in thanking Professor S. P. Niyogi and Dr. Jivraj N. Mehta, the Dean, for their keen interest and encouragement.

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STUDIES IN CALCIUM AND PHOSPHORUS METABOLISM.

Part IV.

THE ABSORPTION OF CALCIUM FROM THE INTESTINE.

BY

R. G. CHITRE,

AND

V. N. PATWARDHAN.

(An Inquiry under the Indian Research Fund Association.)

(Department of Physiology, Seth Gordhandas Sunderdas Medical College, Parel, Bombay.)

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ONCE the food constituents are absorbed from the small intestines, they pass into the general circulation either via the portal venous system or the mesenteric lymphatics, leading to the thoracic duct. According to the older conception the routes which the absorbed food constituents take depends upon the nature of the particular constituent. Since the time of Munk's (1890-91) observation on a patient with lymphatic fistula the opinion has been firmly held that fat leaves the intestine by the lymphatic route. But according to Magee (1930) only 60 per cent of the fat absorbed can be detected in the lymph, the fate of the remainder being still obscure. The work of von Mering (1877), Folin and Denis (1912), van Slyke and Meyer (1912) demonstrated that the absorbed sugar and amino acids took the portal venous route to the general circulation. Recently, however, evidence has been accumulating which goes to show that the transport of the absorbed food constituents may take place simultaneously by both the routes. Hendrix and Sweet (1917) observed that the sugar content of the thoracic duct lymph increased whenever the sugar concentration of blood rose during its absorption from the intestine. Recently, Bolton and Wright (1937) compared the amino-acid nitrogen content of the mesenteric vein with that of lymph from the cisterna chyli and the thoracic duct in dogs during the absorption of peptone and found that the absorption of amino acids from the intestine into the blood capillaries and lymphatics was in accordance with the physical laws of diffusion. There was no clear evidence of selective activity on the part of the blood capillaries or lymphatic endothelium. Patwardhan and Nhavi (1939) have shown that

during the absorption of inorganic phosphate or glycerophosphate from the dog's intestine the inorganic phosphorus content of mesenteric lymph rises with that of the portal blood. So far as the absorption of calcium is concerned only one investigation by Beznák (quoted from Verzár, 1936) is on record from which it was concluded that calcium was not transported via the lymphatics.

EXPERIMENTAL.

Dogs were used as experimental animals. In order to find out the route of absorption of calcium from the intestine to the general circulation, it was necessary to determine the concentration of calcium in the blood from the portal vein as well as in the mesenteric lymph during the absorption of calcium either from food or from some of its soluble salts. The latter can be administered orally or may be introduced intraduodenally under anæsthesia. Both kinds of experiments have been performed and are described below.

The estimations of Ca in blood serum and lymph were carried out according to Wang's (1935) method and inorganic P was estimated according to the method of Bell and Doisy (1920).

ABSORPTION OF CALCIUM WHEN CALCIUM SALTS WERE MIXED WITH FOOD.

A ' fasting sample ' of blood was taken from a peripheral vein in the hind leg of a dog starved for 16 to 20 hours. An intramuscular injection of urethane (1·5 g. per kg.) in water was then given. Immediately after the injection the dog was allowed to eat food in which a known amount of calcium lactate was incorporated. After four hours the abdomen was opened and blood samples from the portal vein were obtained at intervals. These experiments, however, did not give concordant results because it was not always possible to induce the dog to eat all the food and even when a dog ate all the food some of it was not infrequently vomited after an hour or so, rendering quantitative work uncertain. Therefore the procedure of mixing a calcium salt in the food was discontinued. It was decided to introduce a solution of calcium salts (chloride or lactate) intraduodenally under anæsthesia, and then to follow the level of calcium in blood serum and mesenteric lymph.

ABSORPTION AFTER THE INTRADUODENAL ADMINISTRATION OF CALCIUM SALTS.

The dog selected for experiment was starved for 16 to 20 hours before being anæsthetized with either ether, chloroform or urethane. The abdomen was opened and 5 c.c. to 10 c.c. of blood taken from the portal vein exposed by turning over the intestines including the duodenum over to one side.

Lymph, which can be obtained from the thoracic duct or the cisterna chyli, was collected more easily by a method described by Verzár (*loc. cit.*). The lymphatics of the intestines of the dogs meet in the root of the mesentery and form into nodes from which large lymphatics lead to the cisterna chyli. When these lymphatics were cut the lymph oozed out rapidly and could be sucked into a pipette. It was delivered into weighed tubes which were again weighed to find out the quantity

of lymph taken for analysis. This was essential since the lymph clotted rapidly making it difficult to measure the volume accurately. After the fasting samples of blood and lymph were taken, a known amount of a solution of a calcium salt (lactate or chloride), previously warmed to 37°C., was introduced into the duodenum by means of a hypodermic syringe and the abdomen closed by adjusting the flaps and holding them in position by means of forceps. At intervals the abdomen was re-opened and 5 c.c. to 10 c.c. of blood from portal vein and 1 g. to 2 g. of lymph from the mesenteric trunk were taken for analysis. Each experiment lasted for 3 to 4 hours, during which time the dog was kept anæsthetized and warm.

The effect of anæsthesia on Ca and P content of venous blood.

In order to be certain about the validity of results obtained by the above technique, it was necessary to determine the effect of anæsthesia on Ca and P of blood. For this purpose a dog was starved for 16 to 20 hours and a sample of blood withdrawn from a peripheral vein in the hind leg. The dog was anæsthetized with urethane or chloroform and ether and kept under anæsthesia for 3 to 4 hours. At intervals blood was withdrawn from the peripheral vein and serum analysed for Ca and P content. Since the dog was in the post-absorptive stage, there should be no difference in Ca and P content of the portal and peripheral blood, an assumption recently confirmed in this laboratory (Patwardhan and Nhavi, unpublished experiments). Hence it can reasonably be claimed that the determinations of blood from a peripheral vein would give results applicable to the venous blood in general, in the anæsthetized animal. The results of two such experiments are given in Table I :—

Table I.

Effects of anæsthesia on Ca and P content of venous blood.

Particulars.	Time in hours.	Mg. per 100 c.c. blood serum.	
		Ca.	Inorganic P.
(a) Urethane (1·5 g. per kg. of body-weight) anæsthesia maintained by chloroform. Weight of the dog 7·4 kg.	0	12·13	8·02
	3	10·68	8·40
	4½	11·02	9·10
	5½	11·02	10·20
(b) Ether-chloroform anæsthesia. Weight of the dog 10·0 kg.	0	8·73	3·89
	½	8·14	4·60
	1½	8·73	5·40
	2½	8·54	7·37
	3½	8·01	7·80

These and other similar experiments have shown that there is a definite but small decrease of serum calcium under anæsthesia and an appreciable increase of inorganic phosphate. This increase in inorganic phosphate under ether anæsthesia has been noted by Marenzi and Gerschman (1934) and Patwardhan and Nhavi (*loc. cit.*). A fall in serum calcium under the influence of narcotics has been observed by Cloetta, Fischer and van-der Loeff (1934) in dogs receiving paraldehyde or chloral hydrate singly or in mixtures. Other workers, however, are not agreed on this point. Taylor and Caven (1927) found that anæsthesia had no effect on serum calcium. Binet and Blanchetiere (1925), on the other hand, found that in chloral anæsthesia the calcium content of venous blood was higher than that of the arterial blood and that asphyxia was accompanied by hypercalcæmia preceded by a transient hypocalcæmia. Emerson (1928), who used ether as an anæsthetic, also found a 15 per cent increase in serum calcium after anæsthesia and 20 per cent increase in anæsthesia accompanied by asphyxia. He reported, however, a slight decrease in serum calcium following anæsthesia in which there was hyperventilation. Emerson believed that the rise in serum calcium following anæsthesia was probably due to asphyxia.

Any change in the calcium level of the blood of an anæsthetized animal to which a calcium salt has been administered will be the resultant of two factors, viz. the influence of the anæsthesia on blood calcium and the influence of rapid inflow of calcium from the intestine. That the former is small (a slight decrease of 1·0 mg. per cent) has been shown by the above and several such experiments carried out in this laboratory. Hence it may be concluded that any increase in blood calcium that may be observed after the introduction of Ca salt in the duodenum is due to its absorption from the intestine. Having ascertained this, further experiments to find out the route of absorption of calcium were undertaken.

The technique has already been described. Four experiments were carried out using calcium lactate and two using calcium chloride. The concentrations of Ca and inorganic P in blood serum only were determined, since it was thought worth while to investigate the portal venous route first. The results are given in Table II :—

TABLE II.

The absorption of calcium by the portal venous route.

Particulars.	Time in hours.	MG. PER 100 C.C. BLOOD SERUM. Ca.	Inorganic P.
(*a*) Weight of the dog 9·5 kg. 40 c.c. of calcium lactate solution 14·56 per cent containing 756 mg. Ca introduced; pH 7·4.	0	12·03	6·0
	½	12·42	6·16
	1	13·58	7·50
	2	13·39	9·09
	3	13·96	10·27

TABLE II—*concld.*

Particulars.	Time in hours.	Mg. per 100 c.c. blood serum.	
		Ca.	Inorganic P.
(*b*) Weight of the dog 7·0 kg. 40 c.c. of calcium lactate solution 17·36 per cent containing 902 mg. Ca introduced; pH 7·4.	0	13·59	7·62
	½	14·72	7·74
	1	17·06	10·59
	2	16·00	10·81
(*c*) Weight of the dog 8·15 kg. 40 c.c. of calcium lactate solution 7·3 per cent containing 380 mg. Ca introduced; pH 7·5.	0	11·64	6·31
	½	12·80	8·57
	1	12·42	9·33
	2	11·83	9·80
	3	11·83	11·33
(*d*) Weight of the dog 7·25 kg. 40 c.c. of calcium lactate solution 7·3 per cent containing 380 mg. Ca introduced; pH 7·5.	0	8·54	5·78
	½	10·09	6·82
	1	15·52	7·29
	2	15·13	7·51
	3	15·13	8·12
	4	14·74	8·48
(*e*) Weight of the dog 8·2 kg. 25 c.c. of calcium chloride solution 11 per cent containing 1,000 mg. Ca introduced; pH 5·6.	0	11·06	7·72
	½	12·80	8·95
	1	13·58	10·16
	2	13·58	12·29
(*f*) Weight of the dog 7·2 kg. 22 c.c. of calcium chloride solution 11 per cent containing 880 mg. Ca introduced; pH 5·6.	0	12·03	6·80
	½	15·70	7·31
	1	15·85	7·69
	2	18·06	10·22
	3	17·80	12·50

The influence of the orally administered calcium salts on blood calcium has been investigated by various workers. Halverson, Mohler and Bergeim (1917), Clark (1920) and Salvesen, Hastings and McIntosh (1924) were unable to find a rise in blood calcium by feeding calcium salts. But Mason (1921), Stewart and Haldane (1924) and Hoyle (1928), on the other hand, did observe a rise in blood Ca after ingestion of calcium salts. As has been pointed out by Kahn and Roe (1926) the failure to find a rise in blood calcium after ingestion of calcium salts appears to be due to the fact that the analyses were done on isolated blood samples without considering the relationship between the concentration of blood calcium and the time of ingestion. Further, there seems to be a divergence of opinion about the extent and duration of rise in blood calcium following the oral administration (*see* Bauer and Ropes, 1926; Freeman, Kant and Ivy, 1935; Greenberg and Gunther, 1932; Lieberman, 1931).

The experiments reported above (Table II) demonstrate an increase in the Ca level of the portal blood after the intraduodenal administration of calcium salts, although a regular rise and fall in blood calcium was not observed in the present series of experiments. There seemed no relationship between the maximum rise in blood calcium and the amount of calcium introduced per kg. of body-weight. In one case this rise was of the order of only 1 mg. to 2 mg. per 100 c.c. and in others the rise varied between 2·5 mg. and 6·3 mg. per 100 c.c. of serum. The rise in serum calcium seemed therefore to be uninfluenced by (*a*) the dose in relation to body-weight, (*b*) the concentration of the salt introduced, (*c*) the hydrogen ion concentration and (*d*) the pre-experimental level of serum calcium. It is possible therefore to advance this extreme variability of response as a rational explanation of contradictory reports by different authors. All the factors responsible for the control of the level of calcium in the serum seem not yet to be clearly understood.

During the absorption of calcium the inorganic P content of the portal blood also showed a steady rise which, as has been shown before, may be attributed mainly to anæsthesia, because the absorption of the calcium salt which raised the blood calcium might be expected to lower its inorganic P content, the reciprocal relationship between the two elements being well known (Schmidt and Greenberg, 1935).

The experiments described above thus showed that calcium was absorbed via the portal vein and it was now necessary to discover whether Ca could also be absorbed via the lymphatics as well. For this purpose experiments were carried out in which the concentrations of Ca and P in blood from the portal vein and lymph from a mesenteric trunk were determined. The results of these experiments are shown in Table III.

They showed that during the absorption of calcium salt the concentration of calcium in lymph increased along with that in the blood. The amount and the course of increase were practically similar in both the fluids, showing thereby that there was probably no selective activity on the part of the blood capillaries or lymphatics in the absorption of calcium.

TABLE III.

The absorption of calcium by the portal venous system and by the mesenteric lymphatics.

Particulars.	Time in hours.	MG. PER 100 C.C. SERUM.		MG. PER 100 G. MESENTERIC LYMPH.	
		Ca.	Inorganic P.	Ca.	Inorganic P.
(*a*) Weight of the dog 10 kg. 40 c.c. of calcium chloride solution 5·15 per cent containing 750 mg. Ca introduced; pH 5·8.	0	13·39	5·57	13·9	5·20
	½	..	..	15·7	7·40
	1	14·25	6·43	15·10	7·35
	2	17·75	6·70	16·80	7·56
	3	18·62	5·62	18·60	..
(*b*) Weight of the dog 10 kg. 40 c.c. of calcium chloride solution 5·5 per cent containing 800 mg. Ca introduced; pH 5·8.	0	12·29	5·8	12·58	7·24
	1	15·14	5·43	15·10	9·45
	2	15·20	8·16	14·13	8·79
	3	14·22	8·50	13·70	7·60
	4	..	..	12·05	8·91
(*c*) Weight of the dog 9·0 kg. 40 c.c. of calcium chloride solution 5·5 per cent containing 800 mg. Ca introduced; pH 5·8.	0	8·51	6·85	7·24	7·07
	1½	13·18	11·74	12·30	12·30
	2½	..	..	12·74	13·40

The question might arise whether the lymph collected from the mesenteric trunks represented the villus fluid unaltered by admixture with lymph from the intercellular spaces of the rest of the intestinal tissues. Bolton and Wright (*loc. cit.*) have advanced several arguments to prove that during absorption the mesenteric lymph does represent the villus fluid. These can be summarized as follows :—

The tissue fluid is the medium into which the surrounding cells discharge their metabolic waste and from which they derive their supply of oxygen and other substances necessary for their metabolism. This fluid is constantly being renewed from the capillaries. The alterations in its composition are brought about by diffusion of metabolic waste products into the intercellular spaces. These together with water are partially absorbed into venous capillaries, the remainder escaping into the lymphatics. The lymph from the lymphatics therefore represents that portion of the tissue fluid which remains unabsorbed by blood capillaries and is representative of the transuded fluid only when the latter is being rapidly produced.

The lymph from the mesenteric trunk in the fasting state is thus representative of the tissue fluid of the intestinal villi as well as that of the rest of the intestinal tissue and resembles the lymph from other tissues. However, during the process of absorption of foodstuffs from the intestine the villi are flooded with the substances in process of absorption and the lymph escaping into the mesenteric trunk is altered in composition, more by the rapid inflow of the absorbed substance than by diffusion from the arterial capillaries. The pressure gradient from the tissue spaces maintained by the capillaries, the contraction of the muscles of the intestinal wall and the pumping action of villi, all help in driving on the fluid into lacteals as soon as it is formed. The high permeability of the endothelial wall of the lymph capillaries also ensures a rapid passage of substances aided by the factors mentioned above.

It seems unlikely that during this rapid passage down the lacteals the composition of lymph is altered to any measurable extent by the comparatively small output of metabolic waste products. Hence the lymph collected from the mesenteric trunks during absorption does probably represent the villus fluid.

SUMMARY.

1. The absorption of calcium has been studied by introducing calcium chloride or lactate solutions in the duodenum of anæsthetized dogs, and estimating the Ca and P level in the portal blood serum and mesenteric lymph. In preliminary experiments when the dogs were kept anæsthetized for 3 to 4 hours but no calcium salt administered, small but definite fall in serum Ca was observed.

2. Calcium lactate or chloride introduced into the duodenum of anæsthetized dogs caused a rise in the Ca content of the portal blood serum and mesenteric lymph. The increase of Ca in both the fluids indicated that both the portal venous system and the mesenteric lymphatics participated in the absorption of Ca from the intestines.

3. No relationship was observed between the rise in Ca in the portal blood serum and (*a*) the dose or the concentration of calcium salt, (*b*) the pH of the solution introduced or (*c*) the pre-absorption level of Ca in blood serum.

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THE TREATMENT OF SCROTAL ECZEMA, STOMATITIS AND ALLIED CONDITIONS CAUSED BY VITAMIN DEFICIENCY.*

BY

C. O. KARUNAKARAN, M.B., B.S. (Madras), D.T.M. & H., D.P.H. (Camb.), D.B. (Lond.),

AND

P. KRISHNAN NAIR, M.B., B.S., D.G.O. (Madras).

(*Public Health Laboratory, Central Research Institute, University of Travancore.*)

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THE occurrence of sore-mouth, with or without other manifestations of deficiency among persons living on ill-balanced diets, has been reported by a large number of investigators, within recent years. To mention but a few, Fitzgerald (1932) found the prevalence of angular stomatitis and irritative glossitis in a jail in Assam, during the seasons when vegetables were poor in quality. In some of these cases defective absorption due to dysentery or irritation caused by excessive consumption of chillies (*Capsicum annum*) was believed to cause the trouble, while in others it could be cured only by giving one ounce of yeast daily for several days. It was concluded that the disease was early pellagra. Nicholls (1934) noted it in the poorer schools and asylums in Ceylon in association with phrynoderma. Although only 80 per cent of cases of angular stomatitis had phrynoderma and advanced cases of phrynoderma and keratomalacia were seen without angular stomatitis, it was surmised that deficiency of vitamin A was responsible for both phrynoderma and angular stomatitis. Landor and Pallister (1935) reported cases of angular stomatitis and glossitis in 33 per cent of inmates in some of the prisons of Malaya. Cod-liver oil and orange juice had no curative

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action but Marmite and yeast were effective. Wright (1936) reported cases in Sierra Leone, in which angular stomatitis and glossitis were associated with scrotal eczema. The condition improved when vitamin A or B—administered as Marmite—was given, but for complete cure vitamins A and B were both necessary. He also found sulphur effective in some cases. He therefore called the condition 'polyavitaminosis and asulphurosis'.

Aykroyd and Krishnan (1936*a*) recorded the widespread occurrence of xerophthalmia and angular stomatitis among children in a labour camp, living on deficient diet. The same workers (1936*b*) reported cases of sore-mouth together with phrynoderma and xerophthalmia among the boys and girls in two boarding schools. Yeast and skimmed milk were found effective in sore-mouth, but not cod-liver oil. The diet was deficient in flavine-rich foods. Later (1937), they carried out a survey of 24 boarding schools of Southern India and found the incidence rate of angular stomatitis high in institutions having a typical rice diet with very little 'protective' foods. They carried out an experiment (1938) in which alkaline autoclaved yeast, eggs and soya bean were given to groups of children with sore-mouth. Eggs and alkaline autoclaved yeast produced a cure but soya bean was without effect. It was surmised that sore-mouth was due not to flavine deficiency but to deficiency of some other factor in the B_2 complex—probably the P–P factor. Moore (1937) reported cases of sore-mouth and itching scrotum in Nigeria among people whose diet was deficient in milk and proteins of high biological value. In neglected cases, retrobulbar neuritis tended to occur. Marmite brought about a rapid cure of the scrotal condition and the sore-mouth. It was also effective in early cases of retrobulbar neuritis. Bahr and Ransford (1938), reporting a case that was cured on nicotinic acid, stated that the red tongue and excoriation of the angles of the mouth may be regarded as a pre-pellagrous state. This case had diarrhœa and paræsthesia also. Alport, Ghalioungui and Hanna (1938), in treating cases which presented classical signs of pellagra with nicotinamide, observed that chronic atrophic changes of the tongue were only slightly influenced by this treatment.

Sebrell and Butler (1938) produced angular stomatitis and lesions of the lips (cheilosis) by dietetic restrictions in 10 out of 18 women, within 94 to 130 days. Nicotinic acid failed to cure the condition while 0·05 mg. of riboflavin per kilo of body-weight effected rapid cure. One of their cases developed typical signs of pellagra which disappeared when nicotinic acid was given. But while on nicotinic acid (100 mg. daily) she developed angular stomatitis which was cured only after riboflavin was given. Spies and his colleagues (1938) reported 11 cases of pellagra which were cured with nicotinic acid, some of the cases having no signs other than stomatitis and glossitis. They found nicotinic acid of no use in non-pellagrous glossitis. They therefore suggested the term 'ariboflavinosis' for this condition. Oden and Oden (1939) reported cases of 'ariboflavinosis' (angular stomatitis and glossitis) which were cured by 5 mg. of riboflavin daily, in 5 to 12 days. Landor (1939) reported 10 cases of scrotal eczema and/or angular stomatitis which did not improve on nicotinic acid but got cured on Marmite. He concluded that the deficiency is that of some factor in the B_2 complex which is not nicotinic acid. Sulphur was not found effective

in these cases. Sydenstricker *et al.* (1939) reported cases which were known to be chronic pellagrins or which showed evidence of pellagra. The cheilosis and dermatitis were cured by riboflavin, while the patients remained on a pellagra-producing diet. As soon as riboflavin was discontinued there was a tendency to relapse.

Katzenellenbogen (1939) reported 24 cases of glossitis of which 21 showed improvement on the administration of nicotinic acid ; but in the remaining 3 there was no improvement. Aykroyd, Krishnan and Passmore (1939) treated 24 cases having sore-mouth, with or without excoriation of the mucocutaneous junctions and scrotal eczema, with varying doses of nicotinic acid ; 16 of their cases showed definite improvement, while in 8 there was no change in spite of prolonged treatment.

The present investigation.

The medical officer in-charge of one of the large asylums in Travancore sent to us, at the end of July 1939, 3 cases of bad scrotal eczema with a note saying that there were other similar cases in the institution and that he wanted a suitable vaccine to be prepared, various local remedies having been tried in vain. Careful examination showed that the scrotal eczema was associated in many cases with stomatitis and phrynoderma, which are known to be due to diet deficiency. A study of these cases, with a view to discovering methods of curing and preventing the condition by improvements in diet, was undertaken under the auspices of the Research Department of the University of Travancore. The experiments lasted for 10 weeks, from the beginning of August till the second week of October.

The main clinical manifestations were :—

(1) *Dermatitis.*—Involving mainly the scrotum, the groins and the penis. This was the most important and serious manifestation. It was present in 81 of the 92 cases treated and presented an extremely variegated picture. In some, especially the early cases, the skin was red, hot and, in the groins, well elevated above the level of the subjacent skin, with the epithelium almost intact. In others, numerous bleeding or pustular points could be noticed. While the affected area in some had a typical dry powdery appearance, in others it showed copious weeping. A raw, red and often fissured surface left by the desquamated epithelium was the characteristic lesion in some other cases. In a few cases the mucocutaneous junction of the prepuce was pale and fissured. Intense itching was present in all. In 1 case the dermatitis was of an entirely distinct character. This patient had dry scaly elevated patches varying in size from a square millimetre to 4 square centimetres, not only on the scrotum, but scattered all over the body. He was serologically negative for syphilitic infection. A few patients had dry, scaly exfoliation affecting the nasolabial fold and the skin of the nose. Evidence of intercurrent ring-worm infection was present in some (*vide* Plate VIII, figs. 1 and 2).

(2) *Angular stomatitis.*—The angles of the mouth presented a whitish appearance covered with thick moist crusts and on opening the mouth cracks and fissures could be seen. In bad cases the lesions extended to about ½ inch laterally.

The mucous membrane of the lips was redder than usual and, in some, the lips were cracked and dry. Sixty of the 92 cases suffered from angular stomatitis (*vide* Plate VIII, fig. 3).

(3) *Glossitis.*—The tongue was red and smooth and, in a good many cases, the epithelium either on the dorsum or the margins of the tongue was lacerated. These lesions, angular stomatitis and glossitis, made it difficult for the victims to relish their food, particularly curries. Glossitis was present in 58 of the 92 cases treated.

(4) *Phrynoderma* or *hyperkeratosis*, manifesting itself as horny growths affecting chiefly the outer aspects of the extremities and the shoulders, was present in some cases. In the early cases, these growths were about the size of a pinhead, while in more chronic cases they were 3 mm. to 4 mm. in height. Twenty-nine of the 92 cases showed this condition (*vide* Plate IX, figs. 4 and 5).

(5) *Xerophthalmia.*—In some cases the conjunctiva was merely dry and wrinkled, while in others characteristic Bitot's spots were seen. Xerophthalmia was present in 27 cases.

Most of the cases with xerophthalmia complained of defective vision and pain in the eye-balls. The defective vision complained of was, in some cases, of the nature of early night-blindness, while in others it was present even in bright light and there was no associated xerophthalmia. A few of the bad cases were sent to the Ophthalmic Hospital for expert opinion and it was reported that there was nothing abnormal about their retina or fundus, except a slight engorgement of retinal vessels in some.

Bleeding from the gums without any marked degree of pyorrhœa was present in 27 cases. It was said that the bleeding started along with the sore-mouth. None of the cases had diarrhœa. A few complained of burning sensation on the soles and palms. The only case which showed evidence of nerve lesions was serologically positive for syphilis.

Bacteriological examination was carried out in some typical cases. Except in a few cases, where there was a suspicion of intercurrent ring-worm infection, no fungus could be detected. Cultures showed the presence of pyogenic cocci—staphylococcus and streptococcus.

A fairly detailed analysis of the blood in 5 typical cases showed that the red cell count was abnormally low, the lowest being 1·88 millions and the highest 3·5 millions. The colour index ranged from 55 per cent in the former to 80 per cent in the latter. The red cells were found to be larger than normal, the average diameter ranging from 7·2 μ to 7·4 μ (as against 6·9 μ in normal cases) and about 40 to 60 per cent cells had a diameter of more than 7·5 μ. The white cell count was normal, ranging from 5,000 to 12,650. Except for a slight increase in eosinophiles, due to obvious hook-worm infection, there was nothing abnormal about the differential leucocyte count. Even in the cases showing the most acute inflammation of the scrotum the polymorphonuclear cells did not exceed 60 per cent.

In all, 92 cases were treated during this period and of these 30 became available for treatment in the course of the last 4 to 6 weeks of investigation, being fresh cases

Fig. 1. The whole scrotum and the penis are in a dry eczematous condition covered with scabs.

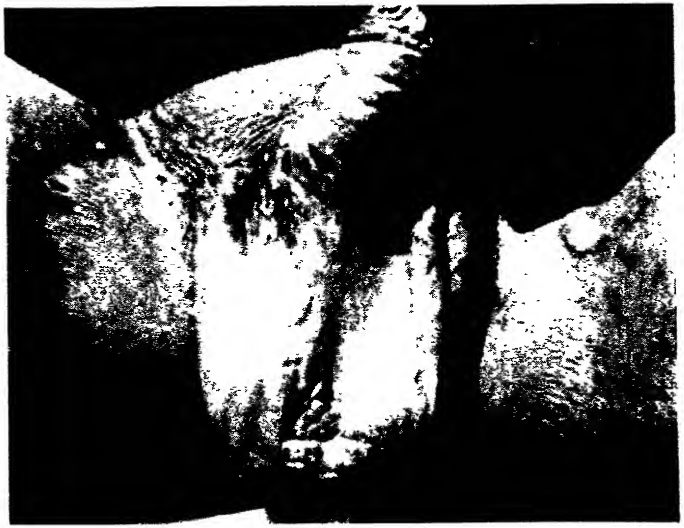

Fig. 2. The skin of the scrotum smooth, red and glistening after extensive desquamation. The groins are also involved.

PLATE IX.

Fig. 4. Phrynoderma of the external aspect of the whole of the right upper extremity.

Fig. 5. Phrynoderma affecting both the forearms.

that occurred in the course of the work. Since the later cases were treated on the basis of the previous findings, the number in each of the groups enumerated below will be found to vary considerably. Fresh cases were treated as they occurred and cases under treatment were discharged as soon as they were cured, or changed to some other group if after two weeks' trial with some particular treatment they did not show any improvement, or if after initial improvement further progress was not noticed for a period of 2 weeks in spite of increased dosage. The period during which individual cases were treated, therefore, varied from case to case. The early cases were divided into 5 groups each having at least 10 cases, as nearly identical as possible as regards clinical manifestations. The first group was given cod-liver oil, 8 c.c. daily, which was raised to 12 c.c. if no progress was noticed. The second group got 3·5 grammes of Marmite—which was doubled in the course of two weeks if progress was not satisfactory. The third group was given a combination of cod-liver oil and Marmite, the fourth got buttermilk equivalent to 8 oz. of skimmed buffalo milk containing 0·5 per cent fat, and the fifth group served as control—being given 5 grains of calcium lactate with sugar. Besides these, 4 persons were given nicotinic acid—150 milligrams per day—for 10 days and 2 were given 0·125 gramme of contramine daily for 6 days to see if, as was suggested by Wright (*loc. cit.*), sulphur deficiency plays any part in the production of these conditions.

Cod-liver oil.—This was given to 18 persons including 1 from the calcium-lactate group; this does not include those to whom it was given for the cure of phrynoderma or xerophthalmia after the other conditions had cleared up. These cases are however noted in brackets (Table I).

TABLE I.

The results of treatment with cod-liver oil.

Clinical condition.	Number.	Cured.	Improved.	No change.
Scrotal eczema ..	16	4	9	3
Angular stomatitis ..	11	..	1	10
Glossitis	11	2	2	7
Phrynoderma ..	6+(3)	5+(3)	1	..
Xerophthalmia ..	6+(6)	3+(6)	3	..

Of the 4 cases with scrotal eczema which were cured on cod-liver oil 1 had no other lesion and although the scrotum and the groins were acutely affected it was

an early case and was cured by 8 c.c. of cod-liver oil daily, in 3 weeks. Two of the remaining cases had phrynoderma in addition to scrotal eczema, while the fourth had xerophthalmia and a mild degree of glossitis. None of these cases had angular stomatitis. Of the 9 cases of scrotal eczema which in the course of a month showed improvement ranging from 50 to 75 per cent but became almost stationary thereafter, and the 3 which showed no progress with cod-liver oil, 9 were completely cured when cod-liver oil was supplemented with Marmite, 2 by Marmite alone after cod-liver oil was stopped, and 1 by buttermilk. The 2 cases cured by Marmite were cases with marked angular stomatitis and showing no other evidence of deficiency. Both of these got 12 c.c. of cod-liver oil daily for 3 weeks with no improvement and subsequently they showed rapid improvement and were cured on receiving 3·5 grammes of Marmite every day for 3 weeks. There was 1 case in this group which had no trouble other than a moderate degree of scrotal eczema, but failed to get completely cured on cod-liver oil. Buttermilk, however, produced a rapid cure.

The 2 cases of glossitis which became normal on being given cod-liver oil were also cases with no angular stomatitis. In 1 case the only other manifestation was severe phrynoderma and in the other case there was scrotal involvement together with xerophthalmia—this being one of the 4 cases of scrotal eczema which got cured on cod-liver oil alone.

The cases of angular stomatitis and glossitis which did not improve on cod-liver oil became normal when given Marmite alone or in combination with cod-liver oil. The cases of xerophthalmia and phrynoderma which did not get completely cured were cases of long duration.

Marmite.—Eighteen cases, including 2 who had cod-liver oil before and 2 from the calcium-lactate group, were given Marmite (Table II).

TABLE II.

The results of treatment with Marmite.

Clinical condition.	Number.	Cured.	Improved.	No change.
Scrotal eczema ..	15	12	1	2
Angular stomatitis ..	10	9	1	..
Glossitis	9	9	..	..
Phrynoderma ..	3	..	..	3
Xerophthalmia ..	2	..	..	2

Of the 3 cases of scrotal eczema which did not get cured on Marmite alone, 2 had cod-liver oil together with Marmite for 2 weeks; on no improvement resulting, they were given buttermilk which effected a cure in both the cases, although in 1 case buttermilk had to be increased to 12 oz. a day before beneficial effects could be noted. The remaining case, although showing only a minor degree of scrotal involvement and no other signs of deficiency, did not respond to 7 grammes of Marmite for 3 weeks, but when 8 oz. of buttermilk was given the condition was cured in 2 weeks. In this group there were 3 cases which showed only extensive scrotal involvement that cleared up completely after Marmite was given for 3 weeks. The case of angular stomatitis which showed only a slight degree of improvement but not complete cure on Marmite was the case mentioned above in which 12 oz. of buttermilk had to be given before scrotal improvement was noticed.

Phrynoderma and xerophthalmia were not improved by Marmite and after the other lesions were cured cod-liver oil was given to these cases. While this had the desired effect, in the course of 2 or 3 weeks some of them began to show relapse in the scrotal condition and the angular stomatitis which was readily controlled either by giving buttermilk or Marmite.

Marmite and cod-liver oil.—Twenty-eight cases inclusive of 5 from the calcium-lactate group, 9 who had received cod-liver oil and 2 who had received buttermilk before, were given Marmite together with cod-liver oil. The 2 cases in the Marmite group who were cured by buttermilk are shown in brackets (Table III).

TABLE III.

The results of treatment with Marmite and cod-liver oil.

Clinical condition.	Number.	Cured.	Improved.	No change.
Scrotal eczema ..	24+(2)	23	1	(2)
Angular stomatitis ..	24+(1)	23	1+(1)	..
Glossitis	21	21	..	..
Phrynoderma ..	12	11	1	..
Xerophthalmia ..	12	11	1	..

The case of scrotal eczema which was not completely cured by Marmite and cod-liver oil had also angular stomatitis, glossitis and bleeding from the gums, all of

which disappeared on this regime. The scrotal eczema also was almost cured, but cracks persisted in the groin without tendency to heal, and he had to be given 12 oz. of buttermilk for 2 weeks before the condition cleared up. But, unfortunately, in this case, the dose of Marmite was not doubled as was done in other cases, where the desired result was not obtained. The case of angular stomatitis which was improved, but not cured, by Marmite and cod-liver oil had bad scrotal eczema together with phrynoderma and glossitis which got completely cured. Even after 6 weeks on Marmite and cod-liver oil a minor degree of angular stomatitis persisted; this cleared up in 2 weeks on 8 oz. of buttermilk. One case in this group is worth special note. He had bad scrotal eczema with early xerophthalmia and slight bleeding from the gums. He made a rapid recovery on Marmite and cod-liver oil and at the end of 3 weeks the Marmite was stopped while cod-liver oil was continued. After a week the scrotal condition recurred. He was then given 7 grammes of Marmite daily, without cod-liver oil, for 2 weeks. There was no improvement. He was then given buttermilk for 2 weeks with no appreciable improvement. Then he was put on 7 grammes of Marmite and 12 c.c. of cod-liver oil as was done at first and his condition rapidly cleared up. The case described earlier, with patches resembling psoriasis not only on the scrotum but all over the body, came under this group. He had to be given large doses of cod-liver oil and Marmite for 6 weeks for the condition to clear up completely.

Buttermilk.—Forty-two cases, including 2 from the cod-liver oil, 3 from the Marmite, 2 from the cod-liver oil plus Marmite and 3 from the calcium-lactate groups, but excluding the case from the cod-liver oil plus Marmite group described above, were given buttermilk (Table IV).

TABLE IV.

The results of treatment with buttermilk.

Clinical condition.	Number.	Cured.	Improved.	No change.
Scrotal eczema ..	34+(1)	31	3	(1)
Angular stomatitis ..	29	26	3	..
Glossitis	22	20	2	..
Phrynoderma ..	12	10	2	..
Xerophthalmia ..	9	7	2	..

Two cases having scrotal eczema, angular stomatitis and glossitis received buttermilk for less than 2 weeks. In these cases, although considerable improvement was noticed, complete cure did not occur. If these cases be excluded, only 1 out of 32 cases of scrotal eczema, and 1 out of 27 cases of angular stomatitis, failed to clear up fully on buttermilk. About the former it may be noted that it was a case with only a minor degree of scrotal eczema which after early improvement did not show further progress on buttermilk, but was rapidly cured by Marmite. This is in direct contrast to what had been said above about a similar case—which failed to improve on 7 grammes of Marmite daily but was cured by buttermilk. The only case of angular stomatitis (excluding the 2 already noted, which received treatment for 2 weeks only) which did not get completely cured on buttermilk had only a small degree of scrotal involvement (which was cured by buttermilk) but marked angular stomatitis, phrynoderma and xerophthalmia. This patient had to be given large doses of cod-liver oil and Marmite before all the lesions cleared up. While some bad cases of angular stomatitis, glossitis and scrotal eczema were rapidly relieved by 8 oz. of buttermilk in 2 or 3 weeks, in general, in the early stages, the rate of progress was slower than in the cod-liver oil plus Marmite group. At the end of a month, however, the results in the group, as a whole, appeared to be equally good and in a few cases decidedly better.

Calcium-lactate or control group.—Of the 10 persons in this group 7 had scrotal eczema, 8 angular stomatitis, 6 glossitis, 5 phrynoderma, and 4 xerophthalmia. Since they did not show any improvement and many became worse, at the end of 2 weeks this treatment was discontinued and they were distributed between the groups already discussed.

Nicotinic acid.—Of the 4 cases on nicotinic acid all had scrotal eczema and angular stomatitis and 3 glossitis. None had xerophthalmia and phrynoderma. They were especially selected to exclude patent vitamin A deficiency. After 10 days, during which a daily dose of 150 milligrams of nicotinic acid was given, no improvement was noticeable and so they were given 7 grammes of Marmite daily with immediate benefit. These cases, however, are not included in the Marmite group.

Contramine.—Organic sulphur in the form of contramine was given in 2 cases, 0·125 gramme being given intramuscularly daily, for 6 days. Both had scrotal eczema, angular stomatitis and glossitis. These cases were selected to exclude obvious vitamin A deficiency and were observed for 4 days after the final injection. Except for a slight improvement in the scrotal condition in one of the cases there was no other change.

During the course of the investigation there was one week in which heavy rains and sudden fall in temperature occurred. It was found that during this week the rate of progress was either very slow or retarded. At another time there was a wave of influenza in the institution and it was noticed that in those who contracted influenza the deficiency conditions failed to improve or became worse, although treatment was being continued uninterruptedly.

DIET OF THE INSTITUTION.

A diet survey extending over a period of 7 days was carried out to find out if the diet was deficient and whether this deficiency was in line with the results of the above experiments. The schedule shown in Table V gives the results of this survey :—

TABLE V.

The diet of the institution.

Article of food.	Daily ration per consumption unit.	Chemical composition.	
	Grammes.		
Rice	675·0	Proteins	76 grammes.
Green gram	36·0		
Buttermilk	45·0	Fat	39 „
Coco-nut	40·0		
Coco-nut oil	15·0	Carbohydrate ..	580 „
Yam (elephant)	42·1		
Ash-gourd	12·7	Calories	3,000 „
Brinjal	9·8		
Broad beans	1·4	Calcium	0·34 gramme.
Cucumber	13·5		
Pumpkin	15·1	Phosphorus	2·49 grammes.
Drumstick	4·6		
Tamarind	13·5	Vitamin A about ..	300 I. U.
Onions	13·5		
Fish (dry)	18·0	Vitamin B_1 about ..	3–400 „
Plantain (green)	40·5		
Snake-gourd	5·4	Vitamin C about ..	20 mg.
Lady's finger	8·1		

The diet is ample as regards calorie requirements. There is a *per capita* daily supply of 6 ounces of vegetables. The vegetables are divided into 2 groups—large and small, in the proportion of 2 : 1. The large vegetables consist chiefly of elephant yam, melons, pumpkins and green plantains, and the small vegetables include drumstick (*Moringa oleifera*), lady's finger (*Hibiscus esculentus*), snake-gourd (*Trichosanthes*

anguina), brinjals (*Solanum melongena*), etc. No leafy vegetables are given. Cucumber also is included among the small vegetables and in the hot months of the year—from April to July—when vegetables such as lady's finger become scarce, the bulk of the small vegetables is composed of cucumber and other vegetables of relatively poor nutritive value. It has been reported that cases of the syndrome described usually occur towards the close of the summer and that an extra ration of 1 oz. of liver for a few days was found effective in most cases. During the season of 1939 the number of cases was much larger—due presumably to the fact that there was a greater scarcity of good vegetables than in previous years owing to severe drought.

The rice consumed was good quality parboiled home-pounded rice retaining a good deal of pericarp. There was a complete absence of symptoms suggestive of vitamin B_1 deficiency. Proteins, while quantitatively sufficient, were almost entirely derived from the rice. Fat intake was on the border of the requirements suggested in Health Bulletin No. 23 (1938). Calcium intake was insufficient. The diet appears to be deficient in vitamins A and B_2.

This is corroborated by the results of the experiments conducted. The existence of xerophthalmia and phrynoderma in about 30 per cent of the cases shows clearly the presence of vitamin A deficiency. Cod-liver oil and buttermilk were effective in the treatment of these cases, but not Marmite. Scrotal eczema, the major complaint investigated, was cured by cod-liver oil in a few cases; but in the majority of cases cod-liver oil by itself was partially or totally unsuccessful. Marmite was of greater effect than cod-liver oil, while a combination of cod-liver oil and Marmite, or buttermilk by itself, was found to give complete relief in almost all the cases. It therefore seems that, while one of the B_2 factors present in Marmite is the chief deficiency concerned in scrotal eczema, a concurrent deficiency of vitamin A also plays a significant rôle in this condition, and that there is a synergic action of both these vitamins as postulated by Wright (*loc. cit.*). It is also possible that the proteins of higher biological value present in the buttermilk may exercise a beneficial effect. In angular stomatitis the vitamin B_2 factor seems to be the only deficiency concerned; in glossitis also this is likely to be the main factor, but probably not exclusively so, because at least 2 cases were cured by cod-liver oil and in some others there was improvement with the same treatment. The bleeding from the gums noted in some cases disappeared along with the cure of the angular stomatitis and glossitis showing the cause of all these conditions to be identical. To judge from the 4 cases on nicotinic acid, it appears unlikely that the factor in the vitamin B_2 complex involved is nicotinic acid. Although only 2 cases were given sulphur, sulphur deficiency does not appear to be involved in these clinical manifestations. The relapses during the cold spell and during the wave of influenza suggest a greater demand for vitamins owing to increased metabolic activity.

Discussion.

Stannus (1936*a*) says, 'Pellagra is a disease which may show great variety in the picture it presents, variety in the course which it takes, variety in the symptomatology, and variety in the individual symptoms. In any single case or small

group of cases any one or more of what have been considered typical symptoms may be absent '........' Of the objective signs angular stomatitis is one of the most characteristic. This condition together with a marginal glossitis is perhaps among the earliest signs and may anticipate more active symptoms by many years.' Eczema of the scrotum is also considered by him characteristic of pellagra. Stannus (1936*b*) is inclined to consider that even phrynoderma, as described by Nicholls (*loc. cit.*) in Ceylon, is pellagrous in character. Phrynoderma is now well recognized to be due to the deficiency of vitamin A. There is, however, a tendency to look upon sore-mouth and scrotal eczema as pellagrous manifestations. If this be justified one would expect nicotinic acid, now accepted to be the P–P factor, would be effective in such cases. But the observations of Landor (*loc. cit.*) and Sebrell and Butler (*loc. cit.*) corroborate the results recorded in this paper regarding the inefficacy of nicotinic acid, while Sydenstricker *et al.* (*loc. cit.*) take the view that even in chronic pellagrins on a pellagra-producing diet, the cheilitis and dermatitis may be due to flavin deficiency. There is, on the other hand, a mass of evidence, reference to some of which has already been made, that the sore-mouth and dermatitis of classical pellagra are easily cured by nicotinic acid. It may be noted that, although Katzenellenbogen (*loc. cit.*) found nicotinic acid useful in 21 out of 24 cases of sore-mouth, these cases occurred in winter when pellagra showed the lowest incidence in Palestine. Aykroyd *et al.* (*loc. cit.*) who have recorded improvement of sore-mouth with nicotinic acid, in a large number of their cases, are of opinion 'that the term pellagra should not be applied to stomatitis and the lesions which sometimes accompany it in ill-nourished rice eaters in India'. One is therefore forced to speculate whether deficiency of both nicotinic acid and flavine may give rise to cheilitis and scrotal eczema, or whether riboflavin and nicotinic acid have a synergic action; or if the co-existence of other deficiencies like the deficiency of vitamin A or of proteins of biological value has any influence on the occurrence of these clinical conditions.

Summary.

1. An investigation has been carried out on an outbreak of scrotal eczema, sore-mouth, xerophthalmia and phrynoderma in an institution in Travancore. Ninety-two persons were given differential treatment with cod-liver oil, Marmite, cod-liver oil plus Marmite, butter milk, nicotinic acid, organic sulphur (contramine) and calcium lactate. The chief complaint, scrotal eczema, appears to be a poly-avitaminosis in which deficiency of some factor in the vitamin B_2 complex other than nicotinic acid is probably the chief deficiency concerned. This factor and vitamin A seem to have a synergic action. The sore-mouth is due almost wholly to the deficiency of the vitamin B_2 factor.

2. Analysis of the diet of the institution showed deficiency of vitamins A and B_2.

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THE QUANTITATIVE ESTIMATION OF NICOTINIC ACID IN BLOOD AND OTHER BODY FLUIDS.

BY

B. D. KOCHHAR.

(Department of Pharmacology and Therapeutics, K. E. Medical College, Lahore.)

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THE recent discoveries of the rôle of nicotinic acid in human nutrition emphasize the need for a method by which the amounts present in blood and other body fluids could be estimated for clinical purposes. Clinicians are often confronted with the problem whether nicotinic acid deficiency is a causative factor in various gastro-intestinal, nervous and skin disorders which do not show the clinical signs of pellagra. Although insufficient intake of nicotinic acid must be very rare among the wheat-eating populace of the Punjab, it is quite conceivable that nicotinic acid 'sub-nutrition' may occur as a result of defective absorption or increased requirement.

There is no specific test for nicotinic acid or its amide. Methods for its estimation are based on the observation of König (1904) that pyridine forms a colour complex with cyanogen bromide and primary or secondary amines; and on the finding of Vongerichten (1899) that pyridine reacts with 2 : 4 dinitrochlorobenzene in alcoholic sodium hydroxide to form a red purple colour. Based on the observation of König (*loc. cit.*) are the methods for estimating nicotinic acid developed by Swaminathan (1938), Shaw and Macdonald (1938) and Bandier and Hald (1939), while the method developed by Vilter, Spies and Mathews (1938) and Karrer and Keller (1938) are derived from Vongerichten's observation.

For estimating small quantities of nicotinic acid or its amide, as in blood, the method which gives the highest colour intensity with a given amount of nicotinic acid will be most suitable. Swaminathan (1938) described a technique for estimating nicotinic acid in blood in which blood proteins were precipitated with tungstic acid and the colorimetric procedure of the cyanogen bromide test applied. Shourie and Swaminathan (1940) have applied the same technique to rat's blood. Pearson (1939) has modified the method and has used it for estimating nicotinic acid in the blood of mammalia, large quantities of blood being obtained for de-proteinization. The author has not tried the method of Vilter, Spies and Mathews (*loc. cit.*) in which 2 : 4 dinitrochlorobenzene is used; according to these

authors the method is not satisfactory for application to body fluids other than urine. Of the other two methods involving the use of metol, that of Bandier and Hald (*loc. cit.*) was abandoned as the colour developed was of less intensity than the colour developed with aniline in the method of Swaminathan (1938) (Table IV). The use of alcoholic aniline as suggested by Shaw and Macdonald (*loc. cit.*) did not give better coloration than the original procedure of Swaminathan (Table III).

In the present investigation the conditions necessary for the development of the maximum intensity of colour with cyanogen bromide and aniline were investigated in order to discover a quick and reliable method for clinical purposes.

Experimental.

A. Reagents.—

(*i*) Cyanogen bromide: An aqueous solution prepared by adding to decolorization 10 per cent A. R. potassium cyanide to a saturated and fresh solution of pure bromine. The solution was about 5 per cent CNBr and was prepared every day before use.

(*ii*) An aqueous aniline solution (2·5 per cent): This was prepared by adding one-fourth of its volume of water to the freshly prepared saturated solution of aniline. The saturated solution was filtered before dilution.

(*iii*) A stock solution of nicotinic acid containing 1 mg. per ml. of nicotinic acid (B. D. H. Laboratory reagent).

(*iv*) A dilute solution of nicotinic acid containing 10μg. per ml. prepared by diluting the stock solution.

(*v*) Sodium hydroxide, 20 per cent, 10 per cent and 1 per cent aqueous solution.

(*vi*) Hydrochloric acid, 31 per cent, 10 per cent and 1 per cent.

(*vii*) Bromthymol blue solution (British Pharmacopœia, 1932, page 517).

(*viii*) Re-distilled ethyl alcohol, 98 to 100 per cent.

(*ix*) Trichloracetic acid, 15 per cent solution.

B. Method.—

In a 50-ml. conical flask oxalated blood (5 ml.) was laked in water (8 ml.) and was stirred thoroughly after the addition of 15 per cent trichloracetic acid (5 ml.). Alcohol (2 ml.) was added to the flask and after ten minutes it was centrifuged or filtered. An aliquot (8 ml. correspond to 2 ml. blood) in a beaker was treated with conc. hydrochloric acid (0·25 ml.), kept in a boiling water-bath for 5 minutes to hydrolyse any amide present and to reduce its volume, cooled and brought carefully to pH 7 (bromthymol blue) with sodium hydroxide. The hydrolysed liquid (not exceeding 9 ml.) was transferred to a 25-ml. flask containing nicotinic acid (10μg. in 1 ml. EtOH). Two ml. of alcohol were then added followed by 8 ml. cyanogen bromide from the burette. After about 3 minutes' shaking, aniline solution (5 ml.) was added and the colour developed was matched with a standard prepared with 20μg. nicotinic acid

diluted to 9 ml. with water using alcohol (3 ml.), CNBr (8 ml.) and aqueous aniline solution (5 ml.) as above. The error due to the slight non-specific tint of the blood filtrate was corrected by subtracting 0·3 from the colorimetric reading of the unknown, setting the standard at 20. For serum, plasma and cerebrospinal fluid the same method is applicable except that the trichloracetic acid used for de-proteinizing cerebrospinal fluid was a 10 per cent solution.

Note.—The intensity of the colour developed with 20μg. of nicotinic acid dissolved in 12 ml. water using 8 ml. CNBr and 5 ml. aniline solution was taken as 100 units in all the subsequent readings.

C. *Discussion of the method.*—

(*i*) *Cyanogen bromide.*—It was observed that about 8 ml. of cyanogen bromide in 25 ml. of the reaction solution containing 5μg. to 200μg. of nicotinic acid gave the best colour; with smaller amounts of cyanogen bromide the intensity of colour developed was less (Table I). Increased temperature lowered the colour intensity. In Table I is also shown the effect of heat when the reaction solution is heated at 75°C. for 3 minutes after adding CNBr to cold or previously heated nicotinic acid solutions:—

TABLE I.

The intensity of the colour reaction under various conditions.

CNBr ml.	COLORATION. Room temperature. Amount of aniline added (ml.).				75°C.
	3	5	8	10	5
1	39	41	44	..	44
3	55	70	86	..	46
5	64	87	95	100	42
7	73	98	100	104	..
8	75	100	102	106	37
10	90	102	105	..	35
12	..	103	..	..	32

(*ii*) Reaction with aniline and the compound formed by nicotinic acid and CNBr.—

(*a*) *Quantity of aniline.*—The intensity of the colour increased with the addition of increasing amounts of aniline until with about 5 ml. of 25 per cent aniline solution the colour reached its maximum. With a further increase in the amount of aniline the slight increase in colour was due to the interference of the colour of the aniline itself produced on standing for 5 to 10 minutes (Table I).

(*b*) *Intensity of colour.*—The colour was directly proportional to the amount of nicotinic acid. Its intensity was maximum when the interval between cyanogen

bromide and the addition of aniline was at least 3 minutes. This interval could be reduced (1 to 2 minutes) if a drop or so of aniline solution was added to the nicotinic acid solution just before the addition of cyanogen bromide. (This observation was made by M. Swaminathan who communicated it to the author.) It is probable that the reaction involved in the formation of the complex of nicotinic acid with cyanogen bromide is a reversible one. The addition of a few drops of aniline minimizes the reverse reaction and makes available for aniline a greater quantity of the complex to give coloration in a given time. However, it was noted that there was no difference in the intensity of the colour and the results were inconsistent. Therefore, it is likely that addition of a few drops of aniline solution does not completely check the reverse reaction, and before the reaction is completed the fading of colour started. The addition of 98 to 100 per cent re-distilled alcohol to a nicotinic acid solution before the addition of cyanogen bromide increased the intensity of colour. It seems that alcohol helps in preventing the backward reaction and the whole of the nicotinic acid complex with cyanogen bromide is available for the reaction with aniline; the reaction being complete in 3 to 4 minutes when the highest peak is reached. The amounts of alcohol for optimum intensity were 4 ml. to 5 ml.; a smaller or greater quantity decreased the intensity as shown in Table II:—

TABLE II.

The comparative intensity of colour with various amounts of alcohol.

Alcohol ml. ..	0	2	3	4	5	6
Coloration ..	100	109	117	120	120	115

The addition of a 4 per cent alcoholic solution of aniline (Shaw and Macdonald, *loc. cit.*) did not help in increasing the colour intensity. Actually, as shown in Table III, it lowered the intensity and the results were inconsistent:—

TABLE III.

The effects of alcoholic aniline on the intensity of colour.

	COLORATION.	
	No alcohol added to nicotinic acid solution.	Alcohol 5 ml. added to nicotinic acid solution.
1. Aq. aniline (5 ml.)	100	120
2. Alcoholic aniline 4 per cent (1 ml.) ..	76	83
3. Alcoholic aniline 4 per cent (5 ml.) ..	95	64

The intensity of the colour produced by this method was compared with the intensity of colour obtained by the method of Bandier and Hald (*loc. cit.*) in which metol is used. However, the colours produced by the two methods were not exactly comparable in a colorimeter, the colour produced with metol being clear yellow, while that produced with aniline contained a slight red tint. In Table IV the Lovibond units obtained by the two methods are given. Minute traces of aniline or metol which were not easily removed from the apparatus even after thorough cleaning gave a faint coloration after the addition of CNBr which, when heated at 75°C. for 3 minutes by Bandier and Hald's (*loc. cit.*) method, was destroyed; thus a part of nicotinic acid content of the testing material was lost. Table V compares the coloration given by 20μg. of nicotinic acid with aniline and metol using a perfectly cleaned apparatus which prevents the development of coloration after the addition of CNBr :—

TABLE IV.

The colour intensity developed with 80μg. nicotinic acid when various reagents are used.

	Lovibond units.	
	Yellow.	Red.
1. CNBr 8 ml. and aq. aniline 5 ml. ..	3·6	0·5
2. " " " ..	3·5	0·5
3. CNBr 1 ml. heated to 75°C. for 3 minutes + metol saturated solution 12 ml.	3·1	*Nil.*
4. As above	3·1	*Nil.*
5. Alcohol 5 ml. + CNBr 8 ml. + aniline 5 ml.	4·0	0·6

TABLE V.

Intensity of colour obtained with aniline and metol.

Solution number.	Colour intensity.	
	With aniline.	With metol.
1	100	78
2	101	77

(*c*) *Potassium dihydrogen phosphate.*—Bandier and Hald (*loc. cit.*) observed that with the addition of KH_2PO_4 (5 ml. of 2 per cent) to the test material (20 ml.) the colour developed by metol and cyanogen bromide is more pronounced. Swaminathan (1939) used phosphate buffer (2 ml.) for estimating nicotinic acid in urine. Aykroyd and Swaminathan (1940) have described the use of phosphate buffer in the estimation of nicotinic acid content of cereals. Table VI shows that the addition of buffer solution of pH 7 (British Pharmacopœia, 1932, page 526) to the nicotinic acid solution did not increase the coloration :—

TABLE VI.

The effect of the addition of buffer solution.

	Coloration.	
	No buffer solution added.	Buffer solution added 2 ml.
Aq. aniline 5 ml.	100	88
Alcohol 5 ml. with aq. aniline 5 ml. ..	116,120	104,107
Alcoholic aniline 4 per cent, 4 ml. ..	95	101
Alcohol 5 ml. with alcoholic aniline 4 ml. ..	64	89

(*d*) *pH.*—The intensity of colour was fairly constant between pH 5 and pH 10. At pH 2 or at pH 12 the intensity decreased, as is shown in Table VII :—

TABLE VII.

The comparative intensity of colour at various levels of pH.

pH.	2	4	5	6	7	8	9	10	12
Coloration ..	70	95	100	100	100	100	101	100	75

(*e*) *Precipitating reagents.*—The use of metaphosphoric acid, trichloracetic acid or tungstic acid for obtaining protein-free blood filtrate had no significant effect

on the intensity of the colour as is shown in Tables VIII and IX. The filtrate obtained with trichloracetic acid was clear even after hydrolysis in an acid medium and did not interfere with the final colour.

TABLE VIII.

The colour intensity with aqueous nicotinic acid after the addition of various reagents for removing protein from blood. The reaction solution in each case was brought to pH 7 (bromthymol blue).

Protein precipitant.	Coloration.	
	i.	ii.
1. Distilled water	100	100
2. Metaphosphoric acid 10 per cent, 3 ml. ..	101	104
3. Trichloracetic acid 10 per cent, 3 ml. ..	105	100
4. Tungstic acid 10 per cent, 3 ml. ..	100	100

TABLE IX.

The amount of nicotinic acid found in dog's blood to which 100µg. nicotinic acid per 10 ml. of blood was added, various protein precipitants being used.

Protein precipitant used.	Nicotinic acid µg. per 10 ml.
1. Metaphosphoric acid ..	128
2. Trichloracetic acid ..	126
3. Tungstic acid ..	118
4. Metaphosphoric acid without addition of nicotinic acid.	24·8

TABLE X.

The effect of various protein precipitants on the estimated amounts of nicotinic acid on human blood.

AMOUNT OF NICOTINIC ACID μG. PER CENT.		
Metaphosphoric acid.	Trichloracetic acid.	Tungstic acid.
288	288	..
240	248	..
300	360	..
280	308	..
300	328	..
..	330	320
..	266	250

(*f*) *Blank.*—When 8 ml. CNBr and 5 ml. aniline solution were added to distilled water in a 25-ml. flask there was no coloration. The faint colour of the blood filtrate neutralized, as previously described, to pH 7, and measured after the addition of 5 ml. of aniline solution (without adding any cyanogen bromide) making the total volume to 25 ml., was fairly constant. It ranged from 2 to 4 units if 100 units were taken as unity for 10μg. of nicotinic acid. This non-specific colour was allowed for by subtracting 0·3 from the reading given by the unknown blood samples setting the standard at 20.

RESULTS AND DISCUSSION.

Students.—Examination of 41 samples of blood from medical college students showed the average nicotinic acid content of blood to be 367 $\pm$ 129μg. per cent (Table XI). These values are of the same order as those recorded by Swaminathan (1938) in three samples of blood, i.e. 530μg., 370μg. and 330μg. per cent.

The average nicotinic acid content of blood serum in five determinations on students was 92 ± 44μg. per cent, ranging from 62μg. to 170μg. per cent.

TABLE XI.

The amount of nicotinic acid in the blood of 41 medical students.

Blood sample.	Nicotinic acid μg. per cent.	Blood sample.	Nicotinic acid μg. per cent
1	456	22	248
2	362	23*	340
3	228	24*	360
4	228	25†	308
5	550	26†	328
6†	467	27	299
7	389	28	320
8	320	29	270
9	346	30	320
10†	456	31	290
11	254	32	220
12	308	33	320
13	310	34	650
14†	360	35*	558
15	311	36	564
16	366	37	316
17	266	38*	847
18†	240	39	565
19	252	40*	207
20	330	41	560
21	228	Mean 367·7 ± 129	

*Light smoker. †Heavy smoker.

In-patients.—The average nicotinic acid content of blood in 18 determinations on in-patients was 355·5 ± 67μg. per cent. The nicotinic acid content of cerebro-spinal fluid, based on four determinations on in-patients, was found to be

$92 \pm 34\mu g.$ per cent, ranging from $56\mu g.$ to $120\mu g.$ per cent. Table XII gives a summary of the results of the estimation of nicotinic acid in the blood ot 18 in-patients suffering from various diseases:—

TABLE XII.

Nicotinic acid in the blood of patients with various diseases.

Blood sample.	Disease.	Nicotinic acid $\mu g.$ per cent.
1	Lupus erythematosus	288
2	Severe anæmia	208
3	,, ,,	348
4	,, ,,	308
5	Nervous disorder	348
6	Muscular dystrophy	356
7	General dystrophy	368
8	Diarrhœa	300
9	Gout	280
10	Dermatitis	384
11	,,	272
12	Lobar pneumonia	400
13	,, ,,	400
14	,, ,,	560
15	,, ,,	468
16	Meningitis	360
17	,,	380
18	N. Y. D.	308
		Mean $355{\cdot}5 \pm 67$

It is important to know the distribution of nicotinic acid in the various constituents of blood. Dorfman, Horwitt, Kaser and Saunders (1939), using the dysentery organism for the quantitative estimation of nicotinic acid, found that all the nicotinic acid activity of blood was in the erythrocytes. Pearson (*loc. cit.*) further observed that mammalia plasma gave with the cyanogen bromide test a colour too faint for colorimetric determination. The findings in Table XIII

agree with the observation of these workers that most of the nicotinic acid is present in the corpuscles. Only whole blood should therefore be used when estimating nicotinic acid.

TABLE XIII.

The amount of nicotinic acid in various constituents of dog's blood.

	Amount of nicotinic acid.			
	Blood μg. per cent.	Serum μg. per cent.	Plasma μg. per cent.	Corpuscles μg. obtained from 100 ml. of blood.
1	288*	36	49†	323
2	268*	..	45	305
3	289	43	68†	253
4	288	30	..	..
5	249	40	60†	200
6	..	46	28	..
7	752	48	16	..
8	520	25	20	..
9	362	30	20	..
Mean	377	36·6	38·2	

*Blood not completely hæmolysed.
†Partial hæmolyses.

If it is assumed that the plasma is 50 per cent volume per volume of blood, the above table shows that only about 5 per cent of the total nicotinic acid in dog's blood is present in the plasma or serum.

SUMMARY AND CONCLUSION.

1. A colorimetric method for the estimation of nicotinic acid in blood, serum, plasma and cerebrospinal fluid has been described.

2. The conditions necessary for the maximum development of the colour have been investigated.

3. The colour developed is directly proportional to the amount of nicotinic acid used in the experiments, i.e. 10μg. to 200μg.

4. The distribution of nicotinic acid in the various constituents of the blood has been studied. It was found that most of the nicotinic acid is present in the cells.

5. The results of 41 estimations of nicotinic acid in samples of blood from students and 18 estimations of in-patients are presented. The mean values are $367 \cdot 7 \pm 129 \mu g.$ and $355 \cdot 5 \pm 67 \mu g.$ per cent, respectively.

6. The level of nicotinic acid in human serum is higher than that in dog's serum.

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A DIET SURVEY OF SOME FAMILIES AND INSTITUTIONS IN CALCUTTA.

Part II.

A NOTE ON THE VITAMIN CONTENT OF THE DIETS.

BY

B. AHMAD,

AND

D. N. MULLICK.

(From the Department of Biochemistry and Nutrition, All-India Institute of Hygiene and Public Health, Calcutta.)

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In the first part of this paper the diet of some middle-class families and institutions in Calcutta was described (Wilson, Ahmad and Mullick, 1936). On account of the lack of adequate information on the vitamin content of common Bengali foodstuffs, the vitamin value of the diets could not be estimated at the time the earlier paper was written. Data about the vitamin content of these foodstuffs are now available from our own studies and those of others. We have, therefore, now calculated the vitamin value of these diets. The results are shown in Table I.

Discussion and Conclusions.

The families included in this study were middle-class Bengali Hindus at three income levels. The number of calories consumed per consumption unit per day shown in the first column reflects the amount spent on food by each family (column 10). In families 1, 4 and 7 in which the number of members is comparatively low and the food expenditure high, the calorie consumption works out to a somewhat excessive figure. In small middle-class families with a high expenditure on food, high figures of calorie consumption might mean a less strict household management and leakage of food.

On the other hand in larger families, at the same level of income, e.g. families 2, 3 and 6, the expenditure on food per head is naturally lower, and the quantity of food consumed falls to the bare minimum, yielding 2,000 to 2,500 calories. The data about the orphanages and the hostels also show a similar relationship between

TABLE I.

The vitamin content of family and institutional diets.

Families.	Description of family or institution.	Total calories.	Vitamin A (μg.).	Carotene (mg.).	Aneurin or thiamin (mg.).	Riboflavin (mg.).	Ascorbic acid (mg.).	Vitamin B_6 (mg.).	Food budget. Total (annas).	On vegetable and fruits, per cent.	On dairy products, per cent.
1	2	3	4	5	6	7	8	9	10	11	12
1	Bengali Hindu family; Approximate income 500/–; Heads 5·125; M. V.* 4·033. Per M. V.* per day	5,924	253·5	25·0	1·9	2·0	199·0	2·76	14·05	40·9	28·1
2	Bengali Hindu family; Approximate income 500/–; Heads 14·0; M. V. 11·85. Per M. V. per day	2,548	237·0	3·3	1·0	0·8	54·9	1·03	6·52	15·7	50·8
3	Bengali Hindu family: Approximate income 500/–; Heads 15·0; M. V. 12·69. Per M. V. per day	2,149	148·8	4·6	0·6	0·6	39·0	0·82	5·46	17·6	48·3
4	Bengali Hindu family; Approximate income 500/–; Heads 8·0; M. V. 7·49. Per M. V. per day	4,212	166·9	11·0	2·4	1·2	112·3	3·20	13·80	41·7	21·5
5	Bengali Hindu family; Approximate income 300/–; Heads 12·0; M. V. 9·59. Per M. V. per day	3,492	88·0	10·0	1·7	0·7	58·1	1·98	3·80	19·9	15·4

6	Bengali Hindu family; Approximate income 300/–; Heads 12·0; M. V. 10·16. Per M. V. per day	1,974	68·8	3·6	0·9	0·5	34·2	1·04	2·89	27·4	38·0
7	Bengali Hindu family; Approximate income 300/–; Heads 6·0; M. V. 4·9. Per M. V. per day	3,850	86·8	20·4	2·2	1·1	118·2	2·28	6·61	44·7	12·3
8	Bengali Hindu family; Approximate income of head of the family 60/–; Heads 7·0; M. V. 6·02. Per M. V. per day	3,248	29·0	6·0	1·4	0·5	39·2	1·39	4·87	26·8	15·6
9	Bengali Hindu family; Approximate income of head of the family 60/–; Heads 11·0; M. V. 9·52. Per M. V. per day	3,366	130·1	14·0	1·7	0·8	73·1	1·81	4·92	40·2	23·2
10	Bengali Hindu family; Approximate income of the head of the family 60/–; Heads 9·0; M. V. 7·56. Per M. V. per day	3,348	191·1	4·0	1·7	0·7	41·1	1·69	3·73	16·8	31·0
	Average for families per M. V. per day.	3,411	141·0	10·9	1·50	0·9	74·9	1·80	6·66	29·1	28·4

* M. V. = Man value or consumption unit.

TABLE I—*concld.*

Families.	Description of family or institution.	Total calories.	Vitamin A (μg.).	Carotene (mg.).	Aneurin or thiamin (mg.).	Riboflavin (mg.).	Ascorbic acid (mg.).	Vitamin B_6 (mg.).	Food budget. Total (annas).	Food budget. On vegetable and fruits, per cent.	Food budget. On dairy products, per cent.
1	2	3	4	5	6	7	8	9	10	11	12
11	Mohammedan orphanage; Heads 202·16: M. V.* 164·00. Per M. V.* per day	2,702	20·2	1·5	1·2	0·7	31·6	1·27	2·68	17·4	0
12	Hindu orphanage; Heads 20·8; M. V. 20·8. Per M. V. per day	2,783	18·5	1·1	1·6	0·6	48·5	1·59	2·96	18·1	2·3
13	Bengali male hostel; Approximate income per head 40/–; Heads 16·0; M. V. 16·0. Per M. V. per day	2,499	26·0	0·6	0·9	0·65	37·7	1·16	3·51	22·9	5·9
14	Anglo-Indian school; Heads 46·0; M. V. 39·8. Per M. V. per day	2,853	32·6	0·6	0·9	0·5	22·5	1·05	4·13	9·43	11·53
15	Marwari gate-keeper; Approximate income 15/–; Heads and M. V. 1·0. Per M. V. per day	5,390	95·9	4·7	1·5	1·4	38·7	1·63	6·78	15·26	23·68
16	Oriya sweeper; Approximate income 10/–; Heads and M. V. 1·0. Per M. V. per day	2,841	7·2	0·8	1·0	0·4	21·4	0·91	2·21	38·70	9·67

* M. V. = Man value or consumption unit.

food expenditure and calorie intake. The Marwari gate-keeper is an exception. Being an athlete and a wrestler he was probably able to supplement his wages from outside sources.

Vitamin A.—The average requirement of an adult person for this factor is approximately 1 mg. if supplied as vitamin A, or 2 mg. or over when in the form of carotene which is not so well absorbed and utilized (Stiebling, 1936; Booher and Callison, 1939). The carotene intake of all the families is high ranging between 5 mg. and 25 mg. per consumption unit per day and much above the requirement. It will be seen from column 11, that those families which have a very high carotene consumption, namely families 1, 4, 5, 7 and 9, spent over 40 per cent of their food budget on vegetables and fruits with the exception of family 5. The consumption of pre-formed vitamin A in all the families is however low. This also applies to families 2 and 3 which spent almost 50 per cent of their budget on dairy products. The vitamin A requirement of all the families, however, is adequately met from the combined intake of vitamin A and carotene.

The carotene intake of the institutions and hostels was, however, inadequate, being only 0·6 mg. to 1·5 mg. per day. Intake of vitamin A was negligible. The same applies to the Oriya sweeper.

Vitamin B_1.—The vitamin B_1 requirement of man has been carefully worked out by Cowgill (1934). The requirement is related to the intake of calories, particularly those derived from carbohydrates, and the weight of the individual. For an average adult person consuming between 2,500 and 3,000 calories, it may be considered to be about 1 mg. (van Veen, 1935; Baker and Wright, 1936).

If this standard is accepted the aneurin intake of the families was adequate with the possible exception of that of family 3. Vitamin B_1 intake per calorie per day, calculated according to Cowgill's formula, is shown in Table II:—

TABLE II.

Ratio of vitamin B_1 intake and calories.

Family.	μg. aneurin per 1 calorie.	Institution.	μg. aneurin per 1 calorie.
1	0·320	11	0·444
2	0·392	12	0·574
3	0·279	13	0·360
4	0·569	14	0·315
5	0·486	15	0·278
6	0·455	16	0·351
7	0·571		
8	0·431		
9	0·505		
10	0·507		
Average for families.	0·439		

The ratios for all the diets were above 0·25, below which Williams and Spies (1938) consider beri-beri will appear. If the ratio 0·25 represents the dividing line between beri-beri-free and beri-beri-producing diets, values only slightly above this ratio, as found in the case of family 3 and the Marwari individual 15, may mean that their vitamin B_1 intake was rather on the low side.

Ascorbic acid.—The ascorbic-acid content of nearly all the diets is high and very much above the reputed minimum requirement of 25 mg. to 30 mg. per day (McCollum, Orent-Keiles and Day, 1939). In those families which had a high food expenditure and spent a large proportion of it on vegetables and fruits, the ascorbic acid intake exceeded 100 mg. (families 1, 4 and 7). Average consumption for other families lay generally between 30 mg. to 50 mg. The vitamin C consumption of the institutions was also of the same order, except that of the Anglo-Indian school, which was low. The diet of the Oriya sweeper, which was the poorest of all, was also the lowest in vitamin C.

It may be pointed out that the ascorbic acid figures shown in Table I are based on the vitamin C content of raw individual foods as purchased. As is well known, the ascorbic acid content of foods is liable to great variations under various conditions, the most important factor being the freshness of the sample. In all green leafy vegetables rapid loss of vitamin C takes place during storage. In the case of most green leafy vegetables, we have found that at room temperatures in Calcutta (32°C. to 35°C.) about 50 per cent of the vitamin is lost in storage during the first 24 hours and as much as 75 per cent in 48 hours. There is again a 25 to 30 per cent loss during cooking. If these losses are allowed for, the estimates of intake must be considerably reduced.

Riboflavin.—The riboflavin intake of the families varied between 0·5 mg. and 2·0 mg. and that of the institutions between 0·5 mg. and 0·7 mg. The intake of the Oriya sweeper was the lowest. Since adequate information on the flavin requirement of human beings is lacking, no observations can be made on the adequacy of these diets with regard to this factor.

Vitamin B_6.—The vitamin B_6 content of the diets has been calculated from results obtained by Wilson and Ray (1938) in this laboratory. Rat units have been converted into milligrams according to the relationship : 1 rat unit = 5 μg. vitamin B_6 hydrochloride. The intake of this vitamin in the various families and institutions was found to lie between 0·82 mg. and 2·76 mg. per man value per day.

Summary.

1. The vitamin A, carotene, aneurin, ascorbic acid, riboflavin and vitamin B_6 content of the diets of 10 middle-class families, 4 institutions and 2 individuals typical of their class, in Calcutta, has been estimated.

The vitamin A content of all the diets was low. The carotene intake of the families was, however, high ranging from 3 mg. to 25 mg. per consumption unit per day. That of the institutions and the 2 individuals was low.

2. Nearly all the diets were found to be adequate in vitamin B_1 in spite of the high calorific value of some of them.

3. Ascorbic acid intake was also adequate except in the case of two diets, those of an Anglo-Indian school and the Oriya sweeper.

4. The riboflavin content of the diets lay between 0·5 mg. and 2·0 mg. and the vitamin B_6 content between 0·82 mg. and 2·76 mg.

5. A close correlation was found between the amount of money spent on food and calorie intake. A similar relationship was also found to exist between carotene intake and the percentage of the food budget devoted to the purchase of vegetables and fruits.

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THE INFLUENCE OF ASCORBIC ACID ON CONTRACTIONS AND THE INCIDENCE OF FATIGUE OF DIFFERENT TYPES OF MUSCLES.

BY

N. M. BASU,

AND

P. BISWAS.

(*From the Physiological Laboratory, Presidency College, Calcutta.*)

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PRESNELL (Presnell, 1934) has shown that during the onset of scurvy the clotting time of blood is markedly delayed. According to Wolbach and Howe (quoted by Eddy and Dalldorf, 1938) vitamin C is responsible for the setting or gelation of a liquid product formed by certain skeletal tissues, such as dentine, bone and collagen of connective tissue. It, therefore, appears probable that vitamin C may behave in the same way with regard to muscles, i.e. may tend to gelate the muscle tissue or membrane, as Ca ion does. It was shown by Gellhorn (1932) that Ca ion delays the onset of fatigue of skeletal muscles as it decreases the permeability of muscle membranes. This effect of Ca ion on the permeability of membranes is probably due to the well-known influence of the former on gelation of living structures (Chambers and Reznikoff, 1925), for the greater the setting or gelation of a structure, the less is the rate of diffusibility of salts or ions through it. Vitamin C also may affect the incidence of fatigue of muscles in the same way as Ca does. This investigation was undertaken with a view to ascertaining how far this supposition was correct.

EXPERIMENTAL.

There being three kinds of muscle in the body, viz. skeletal, plain and cardiac, the influence of vitamin C on each of them was examined as given below:—

I. Skeletal muscle.

The effect of the vitamin on skeletal muscles was studied by the following two methods:—

(*a*) A gastrocnemius muscle of frog was kept immersed in a definite amount of Ringer's solution at pH between 7·2 and 7·4 in a Keith-Lucas muscle trough at

the laboratory temperature and was stimulated for about half an hour by maximal single induction shocks at intervals of 3 minutes and the contractions were recorded on a moving drum on the same abscissa line and with the same point of stimulation. The solution in the trough was then replaced by a fresh solution to which crystalline vitamin C was added so as to form a definite concentration. The actual concentrations of vitamin C used for these experiments were not far removed from what are found in blood and are entered against the curves. The gastrocnemius muscle of the other leg of the same frog was then used and stimulated as in the first case.

This order of experimentation was reversed in another series of curves, i.e. the gastrocnemius muscle of one leg was first stimulated in the presence of vitamin C and later on the same muscle of the other leg of the same frog was stimulated in Ringer's solution without vitamin C.

Two other series of curves were taken, in the first of which the same muscle was first stimulated in Ringer and later on in Ringer plus vitamin C. In the second series the same muscle was stimulated first in Ringer plus vitamin C and later on in Ringer only. The object of experimentation in these different ways was to remove any doubt whatsoever as to the effect of vitamin C on muscle. It will be observed that the concentration of vitamin C in these solutions was not such as to disturb appreciably the pH of the buffered Ringer's solution. The change in pH, if any, was such as could not be detected by an ordinary Cole's potentiometer.

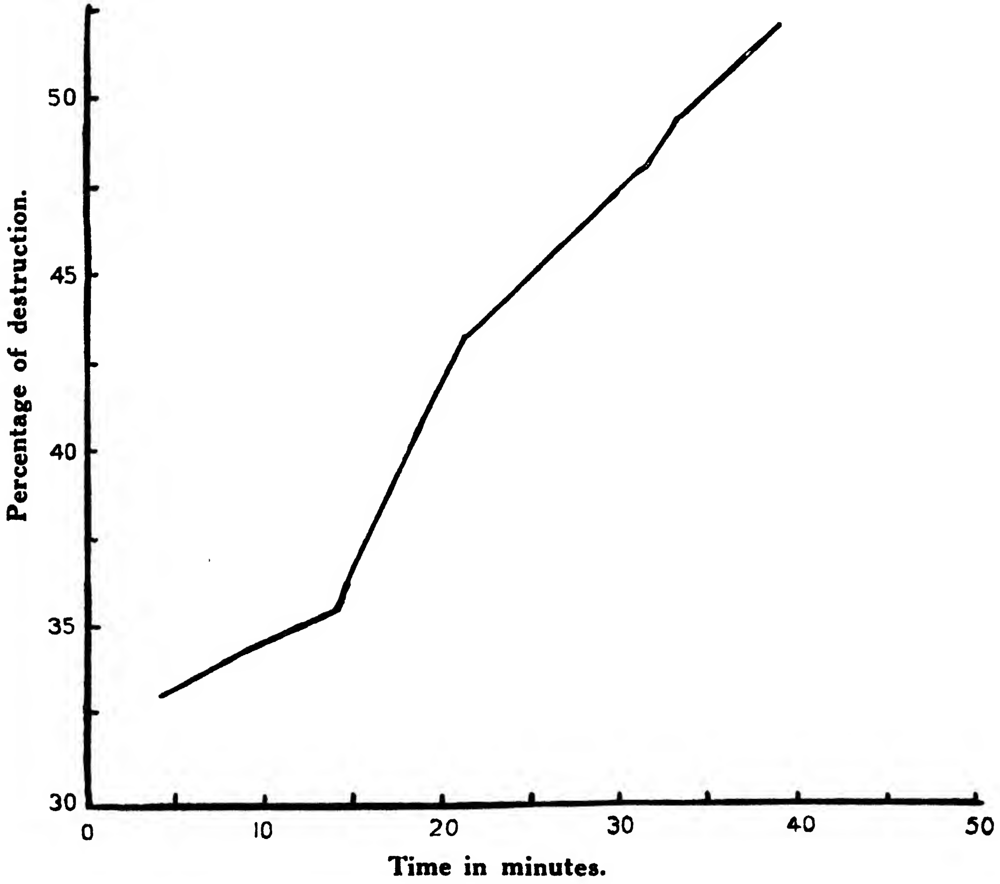
(*b*) Instead of applying single maximal stimuli at intervals of 3 minutes, as in the former series of experiments, the gastrocnemius muscle, placed in a bath of Ringer as in the former case, was subjected to repetitive stimuli from a Neon-lamp stimulation system which was prepared locally under the guidance of the senior author. The advantage of this instrument is that the electrical stimuli delivered from this system are of uniform intensity. The fatigue curve, obtained by these repetitive stimuli, was recorded on a slow-moving drum. The other gastrocnemius muscle of the same frog was then stimulated in the presence of vitamin C and the record was taken. The order of experimentation was reversed, as in the former case, and then the same muscle was for a short period stimulated in Ringer and subsequently in Ringer and vitamin C. The pH of the Ringer's solution was kept between 7·2 and 7·4, Ag-AgCl electrodes were used for stimulating the muscle. These were immersed in the solution a little away from the muscles.

As vitamin C is rapidly destroyed in neutral or alkaline solutions, attempts were made to inhibit its destruction with Na-pyrophosphate (Giri, 1937) but without success. The vitamin was accordingly weighed and added immediately to Ringer just before an experiment to form a sufficiently high concentration, so that, after making an allowance for its partial destruction during the course of the experiment, the amount that was left in the solution was within the limits of concentration required for these experiments. In order that the actual concentration of the vitamin in these solutions at different intervals during the course of the experiment might be ascertained, a solution of known strength of the vitamin in Ringer (the

pH of which was kept between 7·2 and 7·4) was titrated at different intervals with Tillman's dye and the results obtained were plotted out on a graph paper (*vide* Graph 1). The actual concentration of the vitamin at any time during the course of the experiment can be obtained by consulting this curve.

GRAPH 1.

Showing percentage of destruction of vitamin C at pH 7·4 at different intervals of time.



II. Plain muscle.

The effect of the vitamin on plain muscle was studied by ascertaining its action on blood vessels and on intestinal strips.

(*a*) *Blood vessels.*—The effect on blood vessels was studied by the usual vessel perfusion method on guinea-pigs. An animal was killed by coal-gas. After exposing the inferior vena cava an opening was made therein immediately so as to drain out the blood as quickly as possible. A cannula was then introduced into it. Another opening was then made in the arch of the aorta beyond the exit of the three well-known arteries, a cannula connected with a reservoir of Ringer's solution was then introduced through it and the fluid was perfused under great pressure through the vessels so as to wash out blood as completely as possible. The animal was then placed on a horizontal wooden board with the chest upwards. The cannulæ introduced into the aorta and the inferior vena cava are tied and the Ringer's fluid with or without vitamin C was then made to flow through the aorta at a constant pressure. The fluid coming out of the other cannula was led on to a funnel, the stem of which was drawn out to a point which was placed just above two Pt wires sealed to a glass-tube and placed slantingly very near each other, so that as soon as a drop of fluid comes out of the fine opening of the stem of the funnel, it falls in between the two wires and establishes a contact between them. As the wires are slantingly placed, the drop runs down immediately, insomuch that the contacts between the two wires are made and broken as quickly as the drops fall out from the fine opening of the stem of the funnel. These two Pt wires are connected through a thermionic drop-recorder constructed locally under the guidance of the senior author, to an electro-magnetic time-marker which records these contacts by up-strokes of a long lever on a slow-moving drum. A greater number of drops per unit of time indicates relaxation of vessel walls and a smaller number, contraction of the same. Records were taken during perfusion with Ringer alone and with Ringer and vitamin C, the pressure being kept absolutely constant in the same way as is done during heart perfusion (*vide infra*).

(*b*) *Intestinal muscle.*—The effect of vitamin C on the plain muscle of the intestine was studied by Dale's apparatus, its lever being replaced by a simple lever with frontal writing point. Fleisch's solution * was used instead of Ringer. Its pH was kept near about 7·6. The temperature of the bath was kept nearly constant at about 38°C. The bath was ærated at a slow rate throughout the experiment.

The concentration of vitamin C used for studying its effect on intestinal muscle was much greater than that used for skeletal and cardiac muscles and for vessel perfusion experiments. For, the intestinal muscles in the body might be exposed to very great concentrations of vitamin C, depending on the intake.

---

* Fleisch's solution.

Stock solution consists of—

(A) NaCl—210 g., KCl—10 g., $CaCl_2$—6 g., $MgCl_2$—2 g., dissolved in distilled water and made up to 1,000 c.c.

(B) H_3PO_4—3·3 per cent solution in distilled water.

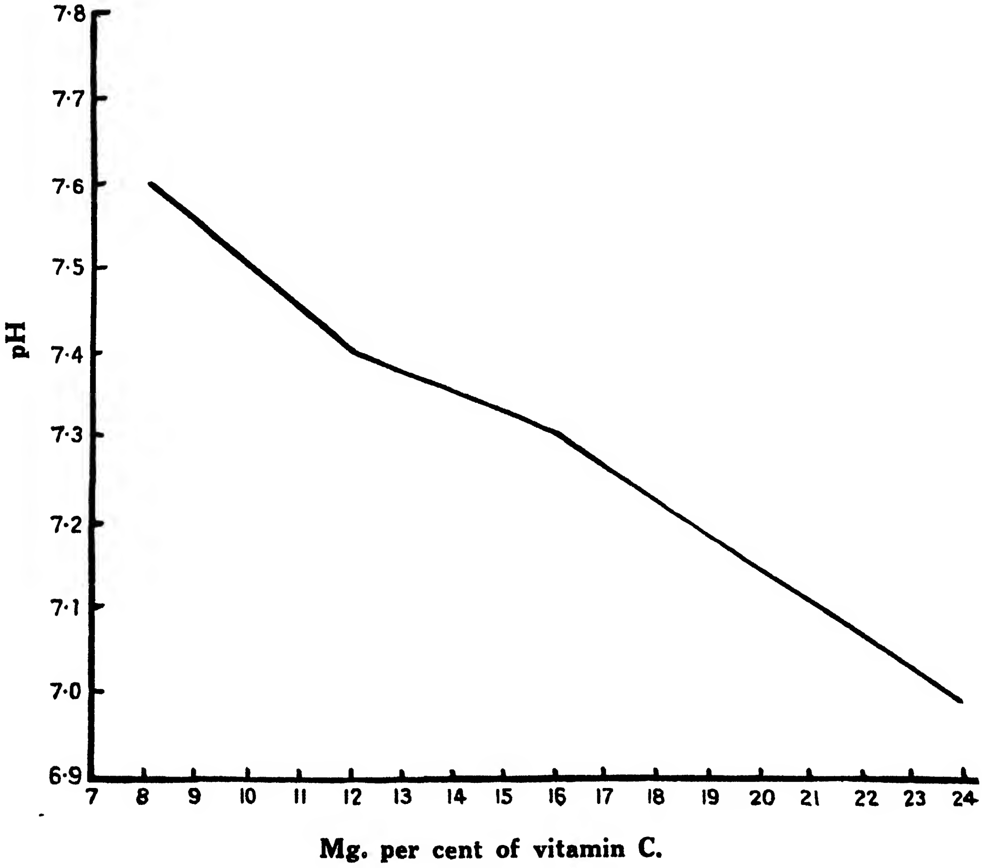
(C) Na_2CO_3—5·3 per cent solution in distilled water.

For use with intestinal muscles, 40 c.c. of (A), 5 c.c. of (B) and 5 c.c. of (C) are made up to 1,000 c.c. with distilled water.

If a large amount of vitamin C be added even to a buffered solution, viz. Fleisch's solution, its pH may change. Accordingly, changes in pH with the addition of different amounts of vitamin C to a definite volume of Fleisch's solution

GRAPH 2.

Showing changes of pH of Fleisch's solution on addition of different amounts of vitamin C.



were ascertained by Cole's direct-reading pH meter and the results were plotted out on a graph paper (Graph 2).

As the effects of high concentrations of vitamin C on the contractility of the intestinal muscle might be entirely due to alterations in pH of the fluid, control curves with Fleisch's solution brought to different pH were taken.

III. Cardiac muscle.

The effect of the vitamin on the cardiac muscle of frog was investigated by the method introduced by the senior author (N. M. B.) in his laboratory. After pithing a frog its heart was exposed. One branch of the aorta was tied and an incision was made into the other branch of the aorta. Through this a cannula, which was bent twice at right angles to its course and drawn out at the end, was introduced, after blood was drained off as much as possible. Another incision was made lower down in the sinus venosus. A cannula for introducing Ringer's solution, or the solution of vitamin C in Ringer, was fitted into the incision and tied to the sinus venosus, taking care that the ligature was applied further away from the sino-auricular junction. The pressure of Ringer's fluid or the solution of vitamin C in Ringer (pH 7·2 to 7·4) was kept constant throughout the experiment by the method adopted in Harris's book (Harris, 1934). The pressure of the perfusion fluid required for maintaining the even flow of liquid through the heart varies between $1\frac{1}{2}$ and $2\frac{1}{2}$ inches according to the size of the frog. This can be easily obtained by raising or lowering the tube provided with a bent side tube, as shown in the diagram of Harris's book mentioned above, so that the lower level of the side tube is at the required height from the level of the heart. The cannula attached to the aorta, being made from a fine tube and being bent twice at right angles in its course, as stated before, offers considerable resistance to the outflow of liquid from the ventricle. In such circumstances the heart beats vigorously and the tracing of the heart-beat is quite prominent. Further, any alterations in the heart-beat due to any drug in the perfusion fluid are easily indicated.

Results.

I. Skeletal muscle.

(*a*) *The effects of application of single stimuli* to a muscle at intervals of 3 minutes when the muscle is immersed in Ringer alone or in Ringer and vitamin C (5 mg. per cent) are shown in Curves 1 and 2. In Curve 1 the muscle was first stimulated several times in Ringer and then in vitamin C solution, but this order was reversed in Curve 2. In analysing these two sets of curves three things become noticeable: (*i*) vitamin C definitely augments the contractions of skeletal muscle, (*ii*) the relaxation period is relatively shortened in the case of vitamin C curves, i.e. relaxation takes place more quickly in the presence of vitamin C and (*iii*) a greater contraction-remainder is generally left in the case of Ringer curves than in vitamin C curves, i.e. relaxation is greater in the presence of vitamin C. Similar results were obtained when the concentration of vitamin C was 4 mg. per cent.

(*b*) *Effects of repetitive stimuli.*—These effects are shown in Curves 3 and 4. The concentration of vitamin was 6 mg. per cent. Similar results were obtained with 4

CURVE 1.

The muscle stimulated first in Ringer and in Ringer plus vitamin C.

1 cm. lengthwise = 0·0183 sec.
pH 7·4

R

Latent period ..	..	0·0183 sec.	
Contraction period	..	0·08784 sec.	difference
Relaxation period	..	0·07137 sec.	0·01647 sec.
Height of contraction	..	5·7 cm.	

Vc (5 mg. per cent)

Latent period ..	..	0·0183 sec.	
Contraction period	..	0·08967 sec.	difference
Relaxation period	..	0·08052 sec.	0·00915 sec.
Height of contraction	..	6·5 cm.	

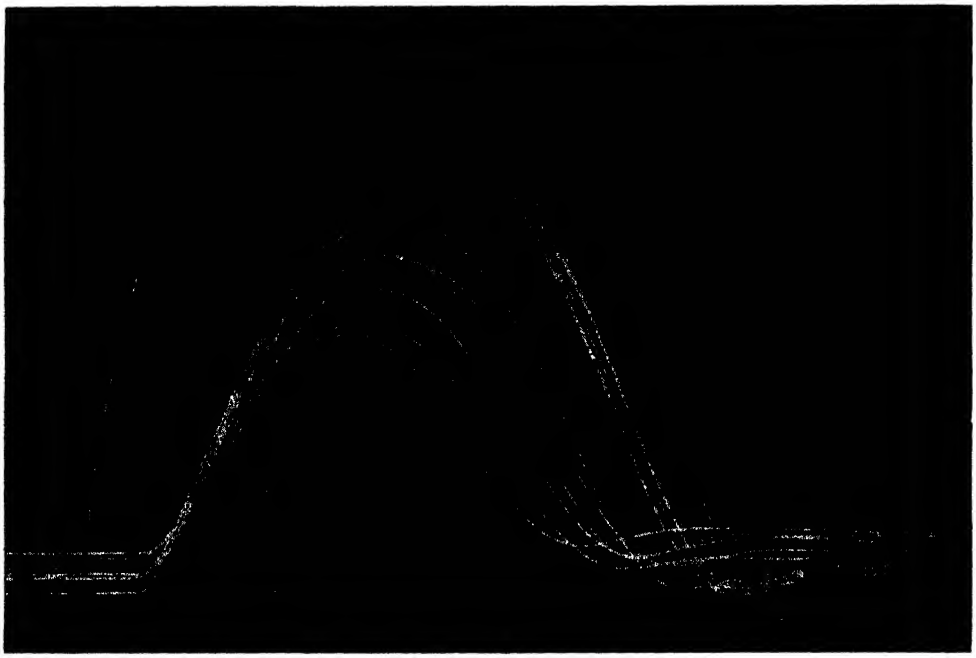
N.B.—Only average values are given.

mg. per cent. The rate of application of stimuli and their strength are exactly the same in both cases. Three points are obvious on comparison of these two curves.

First, the incidence of fatigue is very appreciably delayed in the presence of vitamin C; second, the contraction-remainder is appreciably greater in Ringer than in

CURVE 2.

The muscle stimulated first in Ringer plus vitamin C and then in Ringer alone.



1 cm. lengthwise = 0·0183 sec.
pH 7·4

I.

Vc (5 mg. per cent)

Latent period	.. ..	0·01647 sec.	
Contraction period	.. ..	0·08601 sec.	difference
Relaxation period	.. ..	0·05307 sec.	0·02294 sec.
Height of contraction	.. ..	6·2 cm.	

II.

R

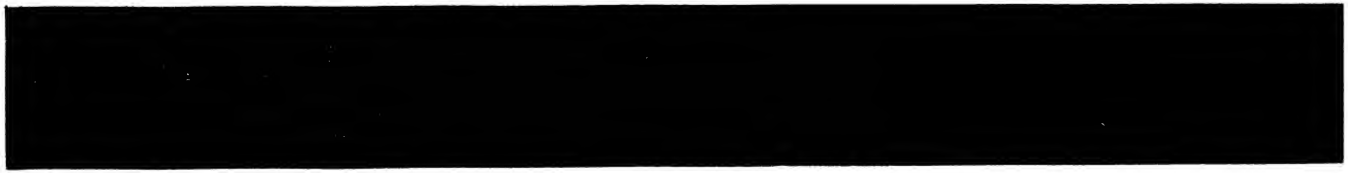
Latent period	.. ..	0·01647 sec.	
Contraction period	.. ..	0·06588 sec.	difference
Relaxation period	.. ..	0·04941 sec.	0·01637 sec.
Height of contraction	.. ..	4·3 cm.	

N.B.—Only average values are given.

vitamin C; and third, the maximum height, i.e. the strength of muscular contraction, is definitely greater in vitamin C than in Ringer.

CURVE 3.

Fatigue in Ringer's solution.



$32 \times \frac{1}{5} = 6{\cdot}4$ sec.
26 stimulation.

3 cm. $\equiv 32 \times \frac{1}{5} = 6{\cdot}4$ sec., i.e. 1 sec. — 0·46 cm.
Stimulation rate :—6·4 sec. — 26, i.e. 1 sec. — 4 times.

CURVE 4.

Fatigue curve in Ringer's solution plus vitamin C.

$34 \times \frac{1}{5} = 6{\cdot}8$ sec.
27 stimulation.

3 cm. $\equiv 34 \times \frac{1}{5} = 6{\cdot}8$ sec., i.e. 1 sec. — 0·44 cm.
Stimulation rate :—6·8 sec. — 27, i.e. 1 sec. — 4 times.

CURVE 5.

Heart-tracing in Ringer's solution and then in Ringer plus vitamin C.

2 mg. per cent.

Weight of toad 40 g.
Mean Ringer height 4·8 cm.

II. Plain muscle.

(*a*) *Blood vessels.*—No appreciable difference could be detected in the rate of outflow of liquid when the vessels were perfused with Ringer alone or with Ringer plus vitamin C (4 mg. or 6 mg. per cent).

(*b*) *Intestinal muscle.*—Curves were taken with intestinal strips in Fleisch's solution at pH 7·6 and after the addition of varying amounts of vitamin C to the solution, i.e. with 5 mg. per cent, 10 mg. per cent, 11 mg. per cent, 12·5 mg. per cent, 20 mg. per cent and 36 mg. per cent vitamin C in Fleisch's solution. Control curves were also taken with intestinal strips immersed in Fleisch's solution of different pH. The analysis of these curves is given below :—

Analysis of curves showing the effects of addition of vitamin C to Fleisch's solution on the contractions of intestinal strips (from rabbit).

Curve number.	Maximum height of contraction in cm. in Fleisch's solution at pH 7·6.	Percentage of vitamin C in mg. in the solution after the addition of vitamin C.	Maximum height of contraction in cm. after the addition of vitamin C.	Alteration in pH after the addition of vitamin C.
1	3·5	5	3·6	Negligible.
2	14·0	10	17·0	7·6 to 7·52
3	5·3	11	7·1	7·6 to 7·45
4	3·8	12·5	5·8	7·6 to 7·4
5	9·6	20	8·0	7·6 to 7·14
6	4·8	36	3·0	Not determined.

Analysis of control curves showing the effects of alterations of pH of Fleisch's solution on the contractility of intestinal strips.

Curve number.	Maximum height of contraction in cm. in Fleisch's solution at pH 7·6.	Alteration in pH of the solution.	Maximum height of contraction in cm. at the altered pH.
1	12·4	7·2	11·9
2	3·1	Between 7·0 and 7·2	3·3

III. Cardiac muscle.

Curves were taken with different concentrations of vitamin C up to 4 mg. per cent which is attained when the body is saturated with vitamin C. An analysis of these curves is given below:—

Curve number.	The height of contraction in cm. when the heart was perfused with Ringer.	Concentration of vitamin C in mg. per cent.	The height of contraction in cm. when the heart was perfused with Ringer and vitamin C.
1	3·6	0·8	4
2	3·9	1·0	4
3	3·8	1·2	5
4	4·8	2	7
5	2·8	4	5·5

DISCUSSION.

The effects of vitamin C on skeletal muscles at from 4 mg. to 6 mg. per cent concentration are exactly similar either when they are stimulated by single induction shocks or repetitive stimuli. These effects are that while it augments contraction it also ensures greater and quicker relaxation and thus delays the onset of fatigue. The behaviour of vitamin C in this respect is similar to that of Ca (Gellhorn, *loc. cit.*).

According to the modern developments of the theory of excitation (Heilbrunn, 1937) of cells or of muscle by such agents as electric shock, mechanical impact, heat, ultra-violet radiation, hypertonic solutions, etc. Ca is released, on excitation, from its combination with protein either in the cell-cortex or in the peripheral portion of muscle tissue. This released Ca causes reversible gelation in the interior of the protoplasm. The assumption of this gelated condition by any tissue is the outcome of excitation. Normally this is caused by the release of Ca as stated above. If Ca is added to the bathing fluid, and if excitation by any of the other methods is made in the presence of this added Ca in the bathing fluid, the gelated condition is expected to be induced more readily and to a greater extent than before and accordingly the response would be stronger than under normal conditions. Further, as the diffusion of a solute through a colloidal substance is slower when it is in a gel. condition than when

it is in a sol. condition, the gelation of the muscle substance will prevent rapid diffusion of lactic acid through the muscle substance produced in the muscle during contractions, particularly during repeated contractions, and will thus delay the onset of fatigue. Vitamin C is known to cause gelation in various fluids (Wolbach and Howe, quoted by Eddy and Dalldorff, *loc. cit.*; Presnell, *loc. cit.*). It is, therefore, very probable that it also causes gelation in muscle plasm. Thus, excitation in the presence of vitamin C will, as in the case of Ca, cause stronger response and, therefore, delayed incidence of fatigue. It is obvious from the results given before that the effects of vitamin C are most prominent in the case of skeletal muscles and least in the case of plain muscle. This can be explained in the following way:—

The refractory period of skeletal muscle is least and of plain muscle greatest. Accordingly, the skeletal muscle can be stimulated very frequently whereas the plain muscle has the slowest rhythm of contractions. When the muscle is stimulated very frequently, Ca released within the muscle in consequence of each stimulation is added on, particularly if the muscle substance be previously gelated, as, for example, by vitamin C, so that Ca may not diffuse back. On account of this increased concentration of Ca a condition more favourable for excitation is set up and accordingly the muscle gives more augmented contractions. In the plain muscle Ca concentrations cannot be increased to such an extent, as Ca released on excitation can diffuse back to a greater extent on account of a longer refractory period in spite of the gelation of muscle substance by vitamin C. The effect of vitamin C, therefore, is not marked. In the case of cardiac muscle the effect of vitamin C is less than that of skeletal muscle but greater than that of plain muscle, as the refractory period of this muscle is greater than that of skeletal muscle and less than that of plain muscle.

In vessel perfusion experiments vitamin C did not produce any tangible results, as these vessels were not stimulated in any way during their perfusion with the vitamin, as was done in the case of other experiments.

Summary.

1. The effect of vitamin C on the contractions of skeletal muscle induced by single and repetitive stimuli was studied. It was found that it augments contraction, brings about quicker relaxation and delays the onset of fatigue.

2. The influence of this vitamin on the rhythmic contractions of heart was investigated and it was found that it augments contractions markedly.

3. The influence of this vitamin on the rhythmic contractions of intestinal muscle is found to be less than that of cardiac muscle or skeletal muscle.

4. An explanation is suggested for the different response of different types of muscle to vitamin C.

5. These effects of vitamin C were studied at such concentrations of vitamin C as are known to occur in the body during saturation. It has also been shown that these effects are not due to any alterations in pH.

ACKNOWLEDGMENT.

We are indebted to Dr. K. Schæfer of Hoffman la Roche for a free and generous supply of standardized vitamin C tablets used in this investigation.

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THE EFFECT OF VITAMIN C ON THE INCIDENCE OF FATIGUE IN HUMAN MUSCLES.

BY

N. M. BASU,

AND

G. K. RAY.

(*Work under the Indian Research Fund Association.*)

(*From the Physiological Laboratory, Presidency College, Calcutta.*)

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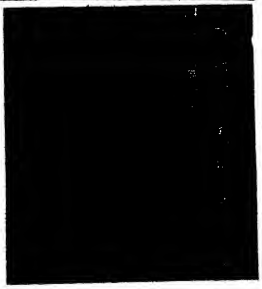
It has been recently shown (Basu and Biswas, 1940) that if frog's gastrocnemius muscles be placed in Ringer's fluid and in Ringer's fluid plus vitamin C (4 to 5 mg. per cent) in a Keith-Lucas muscle trough and be then stimulated by rapidly repeated uniform stimuli delivered from a Neon-lamp stimulation system, the contractions obtained in the latter case are stronger and the onset of fatigue is appreciably more delayed than in the former case. It has been further shown that these effects are not due to alterations in pH that may take place in the Ringer's fluid on the addition of ascorbic acid. This work led the authors to undertake this investigation.

Procedure.

Four persons were selected for these experiments. One of them (N. K. D.) served as a control. The fatigue curve of his finger muscle was taken at a definite hour each day for five consecutive days with Mosso's ergograph. Each of the other three persons (viz. R. K., P. K. B. and S. C. G.) was experimented upon in the following way. The fatigue curve of his finger muscle was taken at a definite hour on the first day. He was given on the next day a dose of 600 mg. of vitamin C tablets and the fatigue curve was again taken one hour and three hours after the ingestion. On each of the following three days he was given a dose of 600 mg. ascorbic acid at exactly the same time of the day and the fatigue curve was taken in the same way. The administration of vitamin C was then stopped, but the fatigue curve was taken at the same hour next day, three days after and five days after the cessation of ingestion of the supplement of vitamin C. The total daily urinary excretion of vitamin C before the ingestion of the supplement, during the period of ingestion and three days after the

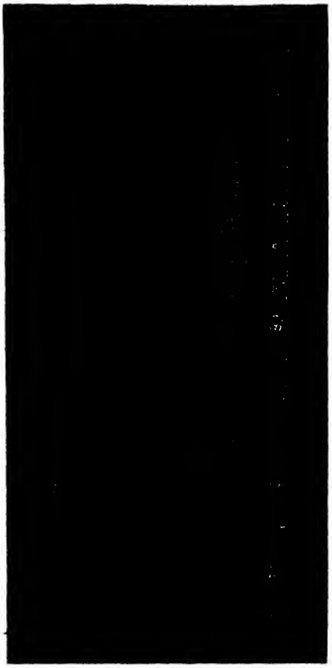
First series of fatigue curves of S. C. G. before and after a daily ingestion of 600 mg. of ascorbic acid for four consecutive days.



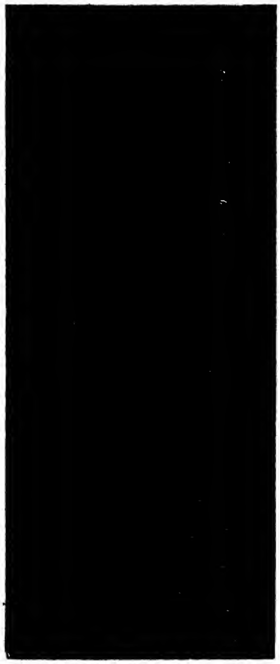
Curve 1.

Curve 2.

Curve 3.

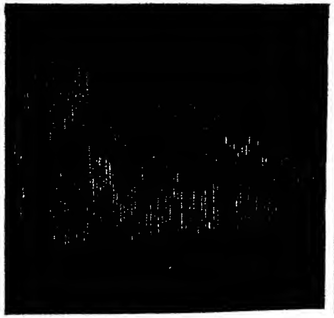


Curve 4.

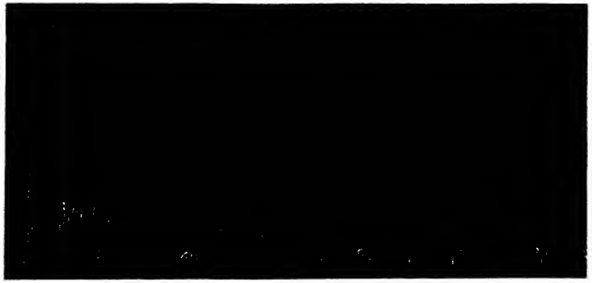


Curve 5.

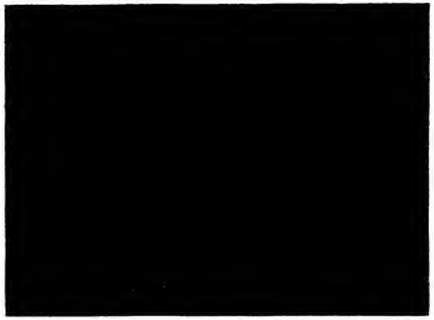
First series of fatigue curves of S. C. G. before and after a daily ingestion of 600 mg. of ascorbic acid for four consecutive days—concld.



Curve 6.

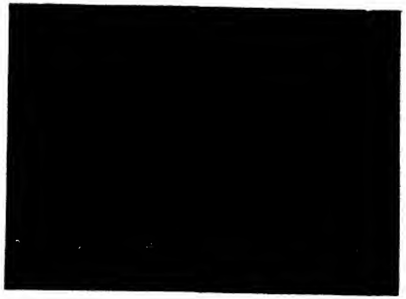


Curve 7.

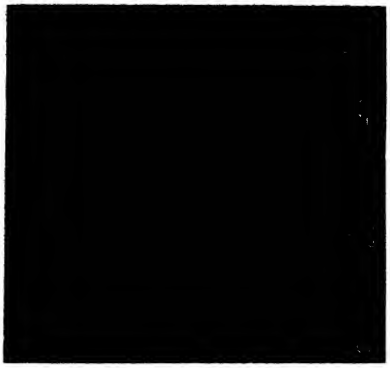


Curve 9.

Curve 8.

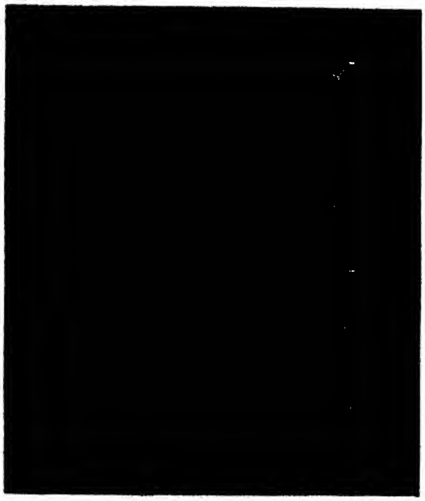


Curve 10.

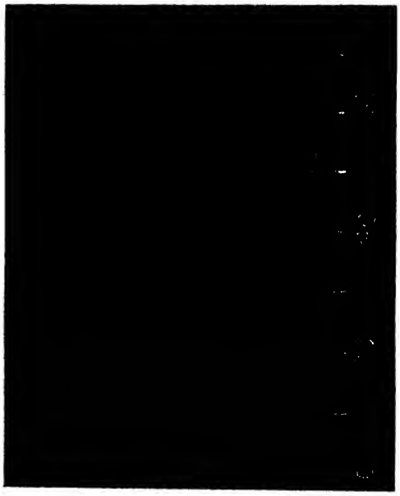


Curve 13.

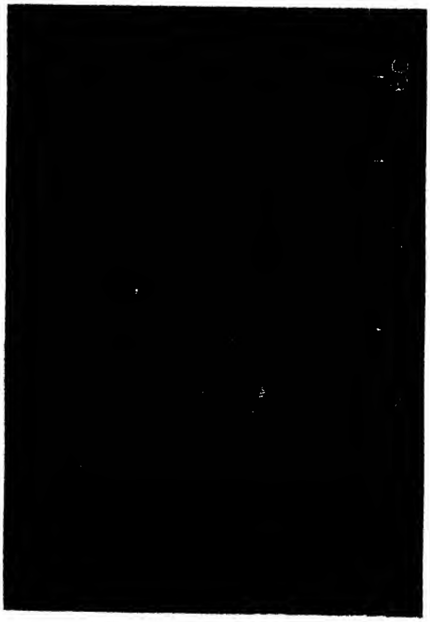
Curve 12.



Curve 11.



Curve 15.



Curve 14.

cessation of the ingestion of the supplement was ascertained. The diet of these four persons, especially with regard to foods rich in vitamin C, and their daily round of work were kept as far as practicable constant during the whole period of the experiment.

Results.

As the results with three persons experimented upon with vitamin C are quite similar, one series of typical curves (Curves 1 to 10) obtained from one of them, viz. S. C. G., are annexed herewith. The curves from the person under control are also attached (Curves 11 to 15). Curve 1 of the first series represents the normal fatigue curve of a finger muscle (viz. that of the middle finger) of the subject (S. C. G.) before any supplement was taken. Curve 2 represents the fatigue curve taken one hour after the first test dose was ingested. Curve 3, taken three hours after the first test dose. Curve 4, one hour after the second test dose. Curve 5, three hours after the second test dose. Curve 6, three hours after the third test dose. Curve 7, three hours after the fourth test dose. Curve 8, 24 hours after the fourth test dose. Curve 9, three days after the fourth test dose. Curve 10, five days after the fourth test dose.

As the difference in the nature of the fatigue curve taken one hour and three hours after the ingestion of vitamin C is not appreciable, as would be obvious from the analysis of Curves 1 to 10 given in Table I, no curve was taken one hour after the third and the fourth test doses of the vitamin.

Table I.

(S. C. G.).

Showing the relationship between the ingestion of vitamin C and the nature of fatigue curve.

Number of curve.		Number of contractions per 10 seconds.	Total duration of the fatigue curve.		Total number of contractions before fatigue sets in.
			Minutes.	Seconds.	
1	First day (without any supplement)	12 or 13	1	10	87
2	Second day (after a supplement of 600 mg. of vitamin C).	14	1	57	164
3		11 or 12	2	15	152
4	Third day (after a supplement of 600 mg. of vitamin C).	12	2	40	192
5		12	2	34	185

N.B.—The maximum height of the normal curve is 42 mm.

The height of the curves after vitamin C ingestion is on the average over 60 mm (maximum height—72 mm.).

TABLE I—*concld.*

Number of curve.		Number of contractions per 10 seconds.	Total duration of the fatigue curve.		Total number of contractions before fatigue sets in.
			Minutes.	Seconds.	
6	Fourth day (after a supplement of 600 mg. of vitamin C).	10 or 11	1	10	74
7	Fifth day (after a supplement of 600 mg. of vitamin C).	10 or 11	2	10	136
8	Sixth day	8	4	3	194
9	Seventh day	15	1	50	165
10	Eighth day	10 or 11	1	46	111

N.B.—The maximum height of the normal curve is 42 mm.
The height of the curves after vitamin C ingestion is on the average over 60 mm. (maximum height—72 mm.).

TABLE II.

(N. K. D.—control).

Showing the nature of the fatigue curve on five successive days.

Number of curve.	Number of contractions per 10 seconds.	Total duration of the curve.	Total number of contractions before fatigue sets in.
11	16	66	105
12	11	65	71
13	13	54	72
14	14	65	91
15	12	75	90

N.B.—The maximum height varies between 45 mm. and 55 mm.

TABLE III.

The total urinary excretion of vitamin C per day before and after a supplement of 600 mg. of ascorbic acid was given each day for four consecutive days.

Subjects.	Before the supplement was given, i.e. normal (mg.).	AFTER THE DAILY ADMINISTRATION OF 600 MG. OF ASCORBIC ACID.				Three days after the ingestion of the last dose (mg.).
		1st day (mg.).	2nd day (mg.).	3rd day (mg.).	4th day (mg.).	
R. K.	6·6	25·0	133·3	200	222	8·2
P. K. B. ..	6·0	12·5	107·0	170	200	7·0
S. C. G. ..	4·6	5·6	33·7	140	166·6	5·0

The second series of curves represent tracings of the fatigue curve of a person on five successive days without any supplement of vitamin C.

A careful analysis of these curves gives the total number of contractions of the finger muscle before the onset of fatigue and the length of time during which these contractions were performed. These are shown in Tables I and II.

Table III gives the total urinary excretion per day of vitamin C before and after the supplement of 600 mg. of ascorbic acid was administered to the three subjects, viz. R. K., P. K. B. and S. C. G., for four consecutive days.

DISCUSSION.

On examining the second series of curves (Curves 11 to 15) it will be observed that the time taken by a person for the onset of fatigue of one of his finger muscles, while the vitamin C status of his body is more or less uniform (the urinary excretion of vitamin C during the five days of the experiment was more or less constant), varies between 55 and 75 seconds, whereas the total number of contractions prior to the incidence of fatigue vary between 71 and 105 seconds. The variation in the maximum height of the curve, i.e. in the strength of contractions, is much smaller, viz. between 45 mm. and 55 mm.

If these results are compared with those of the first series of curves (Curves 1 to 10), the difference is too obvious to be ignored. Thus, it is seen that the total duration of the fatigue curve increased from 1 minute 10 seconds on the first day to 2 minutes 40 seconds on the third day after the ingestion of 1,200 mg. of vitamin C on the second and the third days, although the number of contractions per 10 seconds were nearly the same on both these days. On the fourth day the subject was indisposed and did not take his meals. This was reflected in his fatigue curve. His muscle was fatigued very quickly, viz. 1 minute 10 seconds. He pulled up on the next day and on the following day the total number of contractions of his

finger muscle prior to fatigue were nearly the same as on the third day. After the cessation of ingestion of the vitamin, although his urinary excretion came down to normal amount, neither the duration of the fatigue curve nor the total number of contractions before the incidence of fatigue became normal. This was also observed in the case of the other two persons. This can be easily explained by the observations of Kellie and Zilva (1939) and also by the present authors that the urinary excretion is not always the true index of the saturation of the body with vitamin C and that after the body is saturated, although the urinary excretion of vitamin C may fall down, if the ingestion of the vitamin is reduced, the saturated condition of the body persists for some time.

In what way vitamin C may influence the muscle contractions becomes a matter of considerable surprise and conjecture, particularly when it has been recently shown (Barron *et al.*, 1938; Schultze *et al.*, 1938) that ascorbic acid is not a respiratory carrier in the body.

It has been pointed out in another paper (Basu and Biswas, *loc. cit.*) that the effects of vitamin C on the contractions of excised muscles are similar to those of Ca. It was accordingly argued that vitamin C affects the muscles in the same way as Ca does, viz. that it causes a reversible gelation in the muscle substances. This conclusion is supported by the well-known behaviour of vitamin C in causing the setting or jelling of the liquid product formed by certain skeletal tissues (Eddy and Dalldorff, 1938) and in helping the coagulation of blood (Presnell, 1934).

It is thus obvious that if vitamin C brings about reversible gelation in the muscle substances, it will naturally cause augmented contractions and will delay the incidence of fatigue. Accordingly, the greater the saturation of the body with vitamin C, the more pronounced would be these effects. These results have been actually obtained in the experiments mentioned before.

Conclusion.

From the experiments mentioned above it is clear that the contractibility of a muscle and its fatiguability depend upon the condition of its saturation with vitamin C.

Acknowledgments.

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A CHEMICAL TEST FOR VITAMIN B_6 IN FOODS.

BY

M. SWAMINATHAN.

(*Nutrition Research Laboratories, I. R. F. A., Coonoor, S. India.*)

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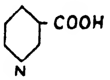
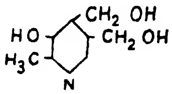
WITHIN the last three years, three members of the vitamin B_2 complex, namely, nicotinic acid (the human anti-pellagra factor), vitamin B_6 (the rat anti-dermatitis factor) and pantothenic acid (the chick anti-dermatitis factor) have been isolated and identified (Elvehjem, Madden, Strong and Woolley, 1937 ; Lepkovsky, 1938 ; Williams and Major, 1940). The chemical formulæ of these factors are as follows :—



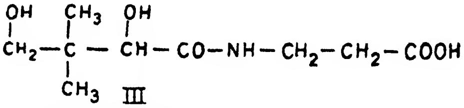
I

Nicotinic acid.



II

Vitamin B_6.



III

Pantothenic acid.

In recent publications from this laboratory (Swaminathan, 1938*a*, *b*) a chemical method for the estimation of nicotinic acid in biological materials, based on a colour reaction with cyanogen bromide and aniline, has been described. Though vitamin B_6 contains the pyridine ring, it does not give the colour reaction with the cyanogen bromide-aniline reagent. In the present paper a chemical method for the determination of vitamin B_6 is described. A preliminary account has already appeared elsewhere (Swaminathan, 1940).

PRINCIPLE OF THE METHOD.

The investigations of Kuhn, Westphal, Wendt and Westphal (1939) and Harris and Folkers (1939) have shown that vitamin B_6 contains a hydroxy group in the β-position of the pyridine ring, which gives the characteristic red coloration of true aromatic phenols with ferric chloride. The vitamin also gives colour reactions with diazotized sulphanilic acid, p-nitroaniline, and the phenol reagent of Folin

and Ciocalteu (1927). The method to be described depends on the use of diazotized sulphanilic acid and p-nitroaniline. Since these reagents are not specific for vitamin B_6, it is necessary to remove all interfering substances before colorimetric estimations can be carried out. The description of the method falls into two parts :—

I. The colorimetric procedure.

II. Its application to test materials.

I. The colorimetric procedure.

Reagents required :—

(1) *Standard vitamin B_6 solution (strong).*—1 ml. = 100 μg. dissolved in N/100 H_2SO_4.

(2) *Standard vitamin B_6 solution (dilute).*—1 ml. = 10 μg. Prepared fresh as required by diluting 1 ml. of solution (1) to 10 ml.

(3) *Sulphanilic acid solution.*—Pure sulphanilic acid (1·5 g.) is dissolved in about 400 ml. of distilled water containing 45 ml. of concentrated hydrochloric acid and diluted to 500 ml.

(4) *P-nitroaniline solution.*—P-nitroaniline (1·6 g.) is dissolved in about 400 ml. of distilled water to which 45 ml. of concentrated hydrochloric acid has been added, and diluted to 500 ml.

(5) *Sodium nitrite* 10 *per cent aqueous solution.*—This solution should be prepared fresh once a month.

All these solutions should be kept in a frigidaire and solutions 3, 4 and 5 in coloured stoppered bottles.

(6) *P-diazobenzenesulphonic acid reagent* (to be prepared fresh just before use).—2·5 ml. of sulphanilic acid solution (1) is measured into a 10-ml. measuring flask. The flask is kept in an ice-bath for about 5 minutes ; 0·4 ml. of 10 per cent sodium nitrite solution is then added. The contents are well mixed and diluted to the mark with ice-cold water and kept on an ice-bath. The solution should be used within two hours of being made up.

(7) *Diazotized p-nitroaniline reagent.*—This is prepared in the same manner as p-diazobenzenesulphonic acid using the same amounts of the various reagents.

(8) *The phenol reagent*—(prepared according to the directions of Folin and Ciocalteu, *loc. cit.*).

(9) Sodium carbonate 5·5 per cent aqueous solution.

(10) Sodium carbonate 20 per cent aqueous solution.

(11) Sodium acetate 50 per cent aqueous solution.

Colorimetric procedures with standard solutions of pure vitamin B_6.

Procedure I. The p-diazobenzenesulphonic acid reagent.—Varying amounts of vitamin B_6 (10 μg. to 30 μg.) are measured into a series of stoppered flasks and each diluted to 10 ml. Four ml. of 50 per cent sodium acetate is then added to each, followed by 1 ml. of the p-diazobenzenesulphonic reagent and the contents well mixed. Two ml. of 5·5 per cent sodium carbonate solution is then added to each and the contents of the flasks well mixed, after which they are allowed to stand for one minute. A bright orange colour develops and reaches its maximum intensity in one minute. A coloured solution containing 20 μg. of vitamin B_6 was kept as the standard with which the other solutions were compared in a colorimeter. The results obtained on comparing various test solutions with the standard were as follows :—

	μg. vitamin B_6.					
Amount taken	10	15	20	25	30	40
Amount determined ..	10	15·5	20	24	30	40

The intensity of colour is proportional to the amounts of vitamin B_6 present. The colour reaction is highly sensitive, 10 μg. of the vitamin being easily estimated. The colour obtained is stable for about five minutes, after which it begins to fade. The estimations must, therefore, be carried out without much delay.

Procedure II. The diazotized p-nitroaniline reagent.—The procedure is exactly similar to the above, except that 1 ml. of the diazotized p-nitroaniline reagent is added in place of the p-diazobenzenesulphonic acid reagent. Results obtained with test solutions were as follows :—

	μg. vitamin B_6.				
Amount taken	10	15	20	25	30
Amount determined ..	9	14	20	24	32

The intensity of the colour produced is approximately proportional to the amount of vitamin present. The colour is stable for more than one hour.

Procedure III. The phenol reagent.—Varying amounts of standard vitamin B_6 solution (10 μg. to 30 μg.) are measured into stoppered flasks and each diluted to 10 ml. Then 0·5 ml. of the phenol reagent is added to each, the contents of the flask being well mixed after the addition. Three ml. of 20 per cent Na_2CO_3 solution is now added to each, the contents well mixed and allowed to stand for 30 minutes. A blue colour develops which reaches its maximum intensity in about 20 to 30 minutes. The solutions are filtered or centrifuged and the clear coloured solutions are used for colorimetric comparison. The coloured solution containing 20 μg. of vitamin B_6 was kept as the standard against which the other solutions were compared. The results of tests similar to those carried out in the case of procedures I and II are given below :—

	μg. vitamin B_6.			
Amount taken	10	20	30	40
Amount determined ..	12	20	28	36

The proportionality of colour was fairly good, but not quite as satisfactory as in the other procedures. The colour is stable for long periods (two to three hours).

Influence of inorganic ions on the colour reactions.—The extracts of foodstuffs, prepared as described later for the colorimetric estimation of vitamin B_6, contain sodium chloride, sodium sulphate and sodium nitrate. If the same solutions are treated with nitrous acid they contain sodium acetate in addition to the above salts. Hence the influence of these salts on the colour reaction was studied. It was found that sodium chloride, sodium sulphate and sodium nitrate in the amounts likely to be present in such extracts (2 to 10 per cent concentrations) had no influence on the colour reaction, while sodium acetate increased the intensity, and affected to some extent the tinge, of the colour produced in the diazo reaction. The maximum effect was obtained with 4 ml. of 50 per cent sodium acetate solution.

II. *Application of the colour reaction to foodstuffs.*

The colour reactions described above are given by other substances besides vitamin B_6. Hence it is necessary to remove all interfering substances before estimations can be carried out. For this purpose the following steps were found to be necessary :—

1. Extraction of vitamin B_6 from foodstuffs.
2. Removal of protein and its derivatives with tungstic acid or lead acetate.
3. Removal of the purine, pyrimidine and imidazole bases with silver hydroxide.
4. Adsorption of the vitamin on 'clarit' acid clay.
5. Elution of the vitamin with hot barium hydroxide.
6. Repetition of silver precipitation, to remove traces of the purine, pyrimidine and imidazole bases that might have escaped precipitation.
7. Concentration to a small bulk (20 c.c.) at pH 6 on a water-bath, the solution being adjusted to pH 7 and made up to a convenient volume (40 c.c.).
8. Estimation of vitamin B_6 present in 10 ml. aliquots using the diazo reaction. As will be shown later, this reaction was found to produce a more satisfactory result than the other procedure using the phenol reagent.

In the sections which follow experiments are described in which variations were introduced in the various stages in order to discover the most satisfactory methods, while the rest of the procedure was kept constant.

A. Preliminary experiments.

(1) *Extraction.*—The investigations of Birch and György (1936) have shown that a greater part of the vitamin B_6 in foods is not extracted by the common solvents. Autolysis or digestion with papain was necessary for the complete extraction of the vitamin present in wheat germ and fish muscle respectively. In the present investigation it was found that digestion of the material with pepsin liberates the vitamin completely. Equally good results were obtained using papain instead of pepsin, while the values obtained after autolysis or extraction with dilute acid (N/10 H_2SO_4) were lower. The values obtained for yeast and rice polishings, using different methods of extraction and applying the procedures described in the sections which follow, are given in Table I:—

Table I.

The extraction of vitamin B_6 from foods.

Vitamin B_6 μg./g.

Methods of extraction.	Yeast.	Rice polishings.
Hydrolysis with pepsin ..	54·0	20·5
Hydrolysis with papain ..	50·8	18·9
Extraction with N/10 H_2SO_4 ..	32·5	13·3
Autolysis	29·2	11·8

(2) *Removal of protein and its derivatives with tungstic acid or lead acetate.*—It was found that lead acetate completely removed protein and its derivatives from plant materials, while with animal tissues and milk complete removal was not effected. Tungstic acid was found to precipitate completely the protein and its derivatives present in all types of material.

Table II shows that the values obtained for the vitamin B_6 content of certain plant materials agree well, whatever the protein precipitant used. With animal tissues, milk and casein, good results were obtained only when tungstic acid was used as the protein precipitant.

TABLE II.

The use of lead acetate and tungstic acid as protein precipitants.

Vitamin B_6 $\mu g./g.$

Foodstuff.	With lead acetate as protein precipitant.	With tungstic acid.
A. *Of vegetable origin*		
Cholam (*Sorghum vulgare*)	8·0	8·5
Maize	9·2	8·6
Yeast	52·0	54·0
Rice polishings ..	18·2	20·5
B. *Of animal origin*		
Liver, sheep ..	Deep interfering colour.	13·3
Muscle, sheep ..	..	4·5
Milk, cow's	..	1·7
Casein (commercial) ..	..	2·2

(3) *Precipitation of purine, pyrimidine and imidazole bases with silver hydroxide.*—The purine, pyrimidine and imidazole bases present in foods interfere in the estimation of vitamin B_6 by the diazo reaction. Vitamin B_1 also gives the reaction under certain conditions (Kinnersley and Peters, 1938; Prebluda and McCollum, 1937). These substances can be quantitatively removed by means of silver hydroxide (Hawk, 1927; Cerecedo and Hennessy, 1937). In order to test whether all the added vitamin B_1 is removed by silver hydroxide, 1 mg. of synthetic vitamin B_1 hydrochloride was added to 2 g. of dried brewer's yeast and the test method, including the addition of silver hydroxide, applied. The following figures show that vitamin B_1 does not interfere in the estimation of vitamin B_6. Further, the value obtained for dried brewer's yeast was the same as that obtained for the same yeast autoclaved in an alkaline medium to destroy vitamin B_1.

Material.	Vitamin B_6 $\mu g.$
Dried yeast, 2 g.	108
Dried yeast, 2 g. + 1 mg. vitamin B_1	102
Dried yeast, 2 g. (autoclaved at pH 9·4)	98

(4) *Adsorption of vitamin B_6 on 'clarit' acid clay.*—Birch and György (*loc. cit.*) reported that vitamin B_6 is adsorbed on Fuller's earth (B. D. H.) and that at least

1 g. of earth to 10 'rat units' (about 50 μg. of vitamin B_6) must be used in order to get anything approaching a quantitative yield. It was found in the present experiments that 'clarit' acid clay is superior to Fuller's earth. Table III gives the results of experiments in which the amount of vitamin B_6 in 2 g. of yeast, and the same quantity of yeast plus 100 μg. and 200 μg. of vitamin B_6 respectively, was estimated using varying quantities of 'clarit'.

TABLE III.

The effect of using varying quantities of 'clarit'.

Amount of 'clarit' used (g.).	Vitamin B_6 found (μg.).		
	Yeast 2 g.	Yeast 2 g. + 100 μg. vitamin B_6.	Yeast 2 g. + 200 μg. vitamin B_6.
1	108	201	250
2	110	208	302
3	112	210	310
4	110	206	315

The values obtained for the vitamin B_6 content of yeast when different amounts of clarit (1 g. to 4 g.) were used, were similar, showing that other substances do not interfere in the estimation; but the recovery of added vitamin was not good when 1 g. of 'clarit' was used to adsorb about 300 μg. of vitamin B_6. Hence, 2 g. of 'clarit' was chosen as the optimum amount for the adsorption of vitamin B_6 from different materials. It is quite likely that 1 g. of 'clarit' would be sufficient when the amount of vitamin B_6 present in the test material is less than 200 μg.

(5) *Treatment of the final extract with nitrous acid.*—Birch and György (*loc. cit.*), and Keresztsy and Stevens (1938) have shown that vitamin B_6 is not destroyed by nitrous acid; it is well known that amino, imino and aromatic phenolic groups, which give the diazo reaction, are destroyed by nitrous acid. In order to discover whether such interfering substances are present in the final extract, the extract was treated with glacial acetic acid (2 ml.) and sodium nitrite (2 ml. of 10 per cent) at 70°C. for 15 minutes. The vitamin B_6 present was estimated before and after treatment, with diazotized sulphanilic acid. The results given in Table V show that no such interfering substances were present in the final extract.

The vitamin B_6 present in extracts treated with nitrous acid could not be estimated with diazotized p-nitroaniline, as the colour obtained was yellowish brown, as compared with the reddish-brown colours obtained with pure vitamin B_6. Further, the presence of some undecomposed sodium nitrite interfered with the estimation when the phenol reagent was used.

B. The application of the method to foods.

A convenient amount (2 g. to 50 g.) of finely minced or powdered test material was suspended in 200 ml. of N/10 H_2SO_4 and heated in a water-bath (70°C. to 80°C.) for half an hour. [An amount of food material containing about 50 μg. to 100 μg. of vitamin B_6, roughly equivalent to 10 to 20 'rat units' of Birch, György and Harris (1935) was usually taken.] The mixture was then allowed to cool to about 38°C.; 0·5 g. of pepsin dissolved in 10 ml. of water and 1 ml. of toluene was then added and the mixture incubated at 38°C. for 24 hours. It was then heated again for half an hour in a water-bath as before, cooled and neutralized (pH 7·2) by the addition of 100 ml. of N/5 barium hydroxide. Next, it was centrifuged and the residue washed with about 100 ml. of water. To the combined centrifugates, 50 ml. of 10 per cent sodium tungstate and 50 ml. of 2/3 N sulphuric acid were added with stirring to precipitate completely protein and its derivatives, the precipitate being removed by centrifuging. The resulting clear centrifugate was adjusted to pH 7·2 by the addition of enough sodium hydroxide (40 per cent), and treated with 70 ml. of N barium acetate to precipitate the sulphuric and tungstic acids present. The precipitate of barium sulphate and tungstate was removed by centrifuging and washed once with 50 ml. of water. To the combined extracts, 15 ml. of N silver nitrate was added, with stirring, followed by 50 ml. of N/5 barium hydroxide, to precipitate completely the purine, pyrimidine and imidazole bases present. The precipitate was removed by centrifuging and the excess of silver and barium present in the centrifugate removed as the chloride and sulphate by the addition of a slight excess of hydrochloric and sulphuric acid, respectively. The precipitate of silver and barium was removed on the centrifuge and the clear centrifugate (if the centrifugate was not clear, it was allowed to stand for half an hour and then filtered through Whatman No. 1 filter-paper) was diluted to about 1 litre. Two grammes of 'clarit' was now added and the vitamin was adsorbed by shaking for five minutes. The 'clarit' was filtered at the pump and washed successively with 50 ml. of N/100 H_2SO_4 and 100 ml. of water and dried in an air oven at 95°C.

In the next stage, 1·6 g. of the adsorbed 'clarit' was weighed into a clean beaker and 60 ml. of N/10 barium hydroxide added. The mixture was heated in a water-bath (70°C. to 80°C.) with stirring for 15 minutes, allowed to cool and centrifuged. The clear supernatant fluid was separated and the residue washed with 20 ml. of water. The combined centrifugates were treated with 3 ml. of N $AgNO_3$ solution to remove any traces of purine, pyrimidine and imidazole bases that might be present. The precipitate was removed on the centrifuge and the excess of silver and barium present was removed as before as the chloride and sulphate. The precipitate

containing the silver and barium was washed once with 20 ml. of water. The combined centrifugate was adjusted to pH 6 and evaporated to 20 ml. on a water-bath. It was finally adjusted to pH 7·2, filtered and made up to 40 ml.

Ten ml. aliquots were used for the estimation of the vitamin B_6 present using diazotized sulphanilic acid and p-nitroaniline, according to the procedure described before, and the colours obtained were compared against that obtained from standard vitamin B_6 (20 μg.) treated in the same manner. The results are shown in Table V.

Estimation of vitamin B_6 using the phenol reagent.—Though the phenol reagent can be used to estimate vitamin B_6 in pure aqueous solutions, it cannot be employed for determinations on extracts from foods prepared as described above, because of the presence of certain interfering substances. The values obtained using the phenol reagent were two to four times those obtained by the diazo reaction, excepting in the case of yeast and potato.

Recovery of vitamin B_6 added to foodstuffs.—To known amounts of various foods 100 μg. of pure vitamin B_6 was added and the procedure described above was followed. The recovery was good in all cases, ranging from 75 to 109 per cent. The results are shown in Table IV :—

TABLE IV.

Recovery of added vitamin B_6.

Experiment number.	Name of foodstuff.	Amount of vitamin B_6 added (μg.).	Total vitamin B_6 found (μg.).	Recovery, per cent.
1	Dried yeast (brewer's) 2 g. ..	..	108	..
	Dried yeast (brewer's) 2 g. ..	100	217	109
2	Dried yeast (brewer's) 2 g. (autoclaved at pH 9·4).	..	106	..
	Dried yeast (brewer's) 2 g. (autoclaved at pH 9·4).	100	188	82
3	Maize, whole, yellow, 10 g. ..	..	71	..
	Maize, whole, yellow, 10 g. ..	100	149	78
4	Muscle, sheep, 10 g. ..	..	45	..
	Muscle, sheep, 10 g. ..	100	120	75
5	Cabbage, 25 g.	..	78	..
	Cabbage, 25 g.	100	167	89
6	Soya bean, 10 g.	..	80	..
	Soya bean, 10 g.	100	178	98

TABLE V.

Vitamin B_6 content of various foods.

Vitamin B_6 $\mu g/g$.

Name of foodstuff.	BEFORE NITROUS ACID TREATMENT.		After nitrous acid treatment using diazotized sulphanilic acid.	Mean.
	Using diazotized sulphanilic acid.	Using diazotized p-nitro-aniline.		
Cereals—				
Cambu (*Pennisetum typhoideum*) ..	10·5	11·4	10·1	10·7
Cholam (*Sorghum vulgare*) ..	8·0	7·5	8·5	8·0
Maize, whole, yellow	7·1	8·8	7·5	7·9
Rice, raw, husked	6·6	7·2	6·8	6·9
Rice, raw, highly milled	3·0	4·0	3·0	3·3
Wheat, whole	7·6	9·2	7·5	8·1
White flour	3·0	3·8	3·1	3·3
Pulses—				
Bengal gram (*Cicer arietinum*) ..	10·4	11·5	10·8	10·9
Black gram (*Phaseolus mungo*) ..	9·6	10·1	10·1	9·9
Green gram (*Phaseolus radiatus*) ..	9·5	11·5	9·2	10·1
Red gram (*Cajanus indicus*) ..	10·5	9·6	10·0	10·0
Soya bean	8·0	9·2	8·5	8·6
Vegetables—				
Beet root	1·3	1·8	1·2	1·1
Cabbage	3·1	2·5	3·0	2·9
Carrots	1·7	2·1	1·8	1·9
Potato	1·5	2·0	1·4	1·6
Flesh foods—				
Liver, sheep	13·4	14·6	13·5	13·8
Muscle, sheep	4·5	5·0	4·4	4·6

TABLE V—*concld.*

Name of foodstuff.	Before nitrous acid treatment. Using diazotized sulphanilic acid.	Before nitrous acid treatment. Using diazotized p-nitro-aniline.	After nitrous acid treatment using diazotized sulphanilic acid.	Mean.
Miscellaneous—				
Milk, cow's	1·7	2·0	1·6	1·8
Rice, polishings	20·5	18·2	18·2	19·0
Yeast, dried (brewer's)	54·0	50·0	50·5	51·8
Yeast, dried (brewer's) (autoclaved at pH 9·4).	53·0	52·0	51·2	52·1

RESULTS.

A sample of dried brewer's yeast was the richest source of vitamin B_6 found; it contained 54 μg. per g. Rice polishings and fresh sheep liver came next in order containing 20 μg. and 14 μg. per g., respectively. Whole cereal grains were estimated to contain approximately 7 μg. to 10 μg. per g. Milled raw rice and white flour contained a little less than half the amount present in the whole cereals, showing that vitamin B_6, like other members of the B group, is concentrated in the outer layers of cereal grains. Pulses are moderately rich in this factor, being equal to whole cereals. Vegetables and milk are poor sources. The proportionate distribution of vitamin B_6 in foodstuffs appears to resemble that of vitamin B_1 and nicotinic acid (Aykroyd, Krishnan, Passmore and Sundararajan, 1940; Swaminathan, 1938*b*).

On the whole the results obtained appear to correspond well with available biological data (Birch, György and Harris, *loc. cit.*; Wilson and Roy, 1938). It is difficult to compare the chemical assays with biological results stated in terms of the 'rat unit', which, according to Birch *et al.* (1935), is the daily amount required to cure 'rat dermatitis' in a period of three weeks, while Wilson and Roy (*loc. cit.*) defined one 'rat unit' as the daily amount which effected a cure in a period of one week. Further, the biological test is complicated by the fact that the production and cure of vitamin B_6 deficiency in the rat are influenced by the amount of unsaturated fatty acids present in the diet (Birch, 1938; Schneider, Steenbock and Platz, 1940).

Discussion.

The chemical method for the estimation of vitamin B_6 in foodstuffs as described should be suitable for application to a wide range of materials containing varying amounts of vitamin B_6. The recovery of added vitamin was good. It was found possible with practice to assay one to two foods per day.

Not much is known regarding the rôle of vitamin B_6 in human nutrition. Spies, Beans and Ashe (1939) have reported dramatic relief of certain symptoms including extreme nervousness, insomnia, irritability and abdominal pain, weakness and difficulty in walking on the administration of 50 mg. of synthetic vitamin B_6 hydrochloride intravenously. The fact that the rat, the dog and the pig develop serious nutritional disorders in the absence of vitamin B_6 (György, 1935; Fouts, Helmer and Lepkovsky, 1939; Chick, Macrae, Martin and Martin, 1938) suggests that it may play an important part in human nutrition. More recently, Smith and Martin (1940) have reported the successful treatment of four cases of cheilosis with synthetic vitamin B_6.

Summary.

1. A chemical method for the estimation of vitamin B_6 in foodstuffs is described. The method is based on the azo colours produced by the vitamin when acted upon by diazotized sulphanilic acid and p-nitroaniline respectively in an alkaline medium. The reaction is highly sensitive, 5 μg. to 10 μg. of the vitamin being easily estimated and the colour developed is strictly proportional to the concentration of vitamin B_6 present.

2. The colour reaction is given by other substances present in foods besides vitamin B_6. The various procedures necessary to remove interfering substances are described.

3. The vitamin B_6 content of 22 foodstuffs has been determined. Dried brewer's yeast was found to be the richest source, while rice polishings and sheep liver came next in order. Cereals and pulses were found to be fairly rich, while vegetables were poor sources of the vitamin.

Acknowledgment.

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LEAD IN FOOD.

BY

RAI BAHADUR K. N. BAGCHI, B.SC., M.B., D.T.M., F.I.C.,

H. D. GANGULY, M.SC., A.I.C.,

AND

J. N. SIRDAR, M.SC.

(Toxicological and Medico-legal Inquiry under the Indian Research Fund Association.)

(From the Department of Chemical Examiner to the Government of Bengal, Calcutta.)

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LEAD is normally present in the urine and fæces of healthy persons and is a normal constituent of all the tissues of our body (Kehoe, Thamann and Cholak, 1933 ; Bagchi and Ganguly, 1937 ; Bagchi, Ganguly and Sirdar, 1939). The routes by which lead is introduced into the system are the alimentary tract, the lungs and the skin. The ingestion of lead with food is a normal process but the amount ingested varies very much according to the nature of foods taken. The absorption of lead through the respiratory tract of those who live in cities, although not connected in any way with any lead industry, is not a negligible factor. In certain occupations necessitating exposure to lead, the lungs and the skin are more important than the other route. The use of lead paints, for example, favours its absorption mostly through the skin and slightly through the alimentary tract but hardly through the lungs. Similarly, external application of medicines and cosmetics containing lead compounds (Monier-William, 1938) and use of red lead on the scalp (hair-parting) by Hindu women (Bagchi *et al.*, 1940) have been known to result in their introduction into the system by absorption through the skin.

ANALYTICAL METHODS.

For this investigation samples of foods, especially those from the vegetable kingdom, were carefully cleansed to eliminate, as far as possible, contamination

with dust, soil and other matters before they were weighed for actual determination, but the process of cleaning could not, for obvious reasons, be carried too far as was done in a previous investigation for determination of lead in human hair (Bagchi *et al.*, 1940). We cannot possibly claim to have obtained the absolute lead figures of the foodstuffs we examined but we have certainly obtained a fairly accurate idea of the amount of lead likely to be ingested in our food in the usual way. About 50 grammes of the sample were taken for each determination and subjected to the usual process of cleansing. In the case of dried fruits and nuts which are exposed for sale on the roadside stalls and are usually full of dust, special care was taken in washing them thoroughly in running water for several minutes and then drying in the sun to their original weight before they were finally weighed for the actual experiment. The dithizone method (colorimetric) of Lynch, Slater and Osler (1934), slightly modified by us as described in a previous communication (Bagchi and Ganguly, *loc. cit.*), was employed in this investigation.

The lead figures given in the tables (see *Appendix*) do not represent the mean of several tests. In most of our samples only one test was carried out but in those yielding no lead or an unusual amount of lead, the experiments were repeated with the same stock of the samples and also with fresh samples from other sources. The meat of goat, for instance, was found to contain much less lead than was found in mutton and, to establish this fact, several samples of both kinds of meat were obtained from different sources and analysed.

The result of analysis of about 160 different kinds of Indian foods, beverages and accessory food materials has been tabulated and shown in the *Appendix*.

Sources of lead in food.

It is comparatively easy to determine accurately the intake of lead through the alimentary tract if the lead content of various foodstuffs is known. Lead in food may be either a normal constituent or of extraneous origin. The use of lead compounds for protection of fruits and vegetables against insect-pests is practically unknown in this country but the use of carbonate, chromate and oxides of lead in paints and colour wash for modern buildings is quite popular even in villages. Such preparations crumble, in course of time, into fine dust which contaminates the soil, water, vegetables and other foodstuffs. The use of lead-containing solders, enamels and glazes in cooking utensils and in vessels in which food is stored, is another source of extraneous lead. Lately, sophisticators have taken to using red lead and other lead pigments to improve the appearance of foodstuffs of inferior quality. The common cereals such as arhar dhal (*Cajanus indicus*) and masoor dhal (*Lens esculentum*) are, for instance, found polished with red lead or lead chromate to make them look like first-grade dhals. Such possibilities are, however, not common in villages where people even now lead, more or less, a primitive life.

Lead in dust.

Thorough cleansing of materials for such investigations is considered essential on account of the fact that dust, particularly in industrial cities, is very rich in lead.

An idea of its lead content was obtained in a previous investigation (Bagchi *et al.*, 1940), by determining the amount of lead in hair collected from the dusty floor of a hair-cutter's shop. In a particular sample, as much as 4 mg. of lead were found in dust contained in a kilo of hair. Blank experiments for checking lead contamination in reagents and glassware, carried out in dusty beakers and flasks, purposely as an experimental measure, gave lead figures much higher than those given by the same glassware after being rendered chemically clean.

Manley has reported that 534 mg. to 12,340 mg. (or 0·534 g. to 12·34 g.) of lead could be obtained from a kilo of dust collected in industrial areas in Leeds. It finds its way into the air from the combustion of low-grade coals and also as a result of various industrial processes (Manley, 1937).

Lead in soil and water.

The lead content of soil and of the water-supply of Calcutta and its neighbourhood was determined with a view to find out to what extent they are responsible for lead in food. The soil of Calcutta or for the matter of that of Bengal, being entirely of alluvial origin, was found to contain a small amount of lead (0·04 mg. to 0·06 mg. per kilo) in comparison with the findings (0·6 mg. to 6·0 mg. per kilo) of the American workers (Kehoe *et al.*, *loc. cit.*). The low lead figures in foods of vegetable origin are therefore comprehensible on the basis of this observation, and it may be presumed that soil contributes only a small fraction to the total lead content of dust, especially in industrial areas. In the country side, the amount of lead in dust may, on the other hand, be expected to be of the same magnitude as of the soil itself—the extraneous sources being negligible. The drinking water supplied by the Calcutta Corporation is also very poor in lead (0·002 mg. per litre)—about one-fifth of that recorded by Kehoe *et al.* (*loc. cit.*) in America or one-tenth of that of the water-supply of Glasgow (Tompsett, 1936) or one-fifteenth of that of London water (Monier-William, *loc. cit.*). Other sources of drinking water, such as tube-wells and tanks in Calcutta and its suburbs, contain only a trace of lead (*vide* Table II—*Appendix*).

Lead in milk and other fatty foods.

Milk and other fatty foods appear to show a discrepancy in their lead content which requires further elucidation. Most of them are rich in lead but the sources from which they are obtained may not contain any lead. Coco-nut oil, for example, yields as much as 1·32 mg. per kilo, while mature coco-nuts do not contain any. A similar relation appears to exist between milk and ghee. No lead could be detected in cow and buffalo milk but ghee from these sources yielded fairly high lead figures (*vide* Table I-G—*Appendix*). The presence of lead in these prepared foods is obviously due to contamination during the process of manufacture and storage. The lead content of human milk appears to vary very much. Out of seven specimens (five from Bengali and one each from Punjabi and European suckling mothers), two specimens from Bengali mothers did not contain any lead, while the remaining three from the same source yielded 0·14 mg. to 0·17 mg. of lead

per kilo. The other two specimens (Punjabi and European) gave 0·01 mg. and 0·04 mg. only. This variation is possibly due to difference in the concentration of lead in the blood which depends on their dietaries, habits and other factors.

It appears that fatty foods are more liable to contamination by lead than other foods under the same conditions of preparation or storage. It is likely that the fatty acids set free from imperfectly prepared or rancid fats and oils readily take up lead either from dust or from the containers and form lead soap. A chemical change of this nature cannot possibly take place in other foodstuffs.

Lead in cooked food.

It may be observed in this connection that lead found in samples of raw food materials is not likely to represent the amount of lead which they would show if cooked and made ready for the table, as for instance, certain specimens of vegetables, fish, etc., having no lead gave appreciable amounts of lead when they were cooked according to Indian methods of cooking, with ghee, oil, salt, turmeric, pepper and other spices (*vide* Table III—*Appendix*). On the other hand, certain kinds of fish having a large amount of lead, gave distinctly lower figures when they were cooked in the same way. In the former case, lead from the accessory food materials (ghee, salt, spices, etc.) was added to foods which contain no lead and in the latter case the foods containing much higher amount of lead got diluted, so to say, by the same accessory materials containing much less lead. Besides the contamination of food with dust during cooking and storing is an inevitable factor partly due to the weather conditions prevailing in this country and partly to habits of the people. In order to determine the lead intake in our food we must, therefore, take into consideration the lead content of cooked food which is actually taken by an individual.

Selective action of animal and vegetable tissues.

In the course of this investigation it has been noticed that different vegetables vary very much in their lead content, from nil to 0·35 mg. per kilo, and that certain kinds of vegetables grown in a particular plot of land differ widely in their lead figures from other kinds growing in the same plot and having the same kind of nutrition. Samples of celery and lettuce, for instance, gave no lead, while cabbages from the same source gave as much as 0·24 mg. of lead per kilo. From this observation it may be suggested that the tissues of a particular plant show a selective action towards lead or, in other words, an affinity for lead which is not possessed by others. In these cases the nutritive soil or water-supply cannot possibly explain the difference in the functions of the vegetable cells. This is also noticed in the animal kingdom. Certain species of fresh-water fish and sea fish extract from water appreciable amounts of lead and retain it in their tissues, while others do not appear to possess any such function. The difference in their diets may, to a certain extent, be responsible for this phenomenon in the case of fish and other animals.

Summary.

1. Lead is present in appreciable amounts in many of our foods both of animal and vegetable origin, while it is absent in an equally large number of foodstuffs from both these sources.

2. Food is liable to contamination with extraneous lead from various sources, viz. solder, enamels or glazes of storing vessels and cooking utensils, soil, water and dust. Dust, especially in industrial areas, is a fruitful source of lead contamination.

3. Tissues of certain animals and vegetables appear to possess a selective action in taking up lead. Some vegetables retain lead while others do not, although they get their nutrition from the same source.

4. Milk and other fatty foods show a discrepancy in their lead content. Ghee contains a fair amount of lead, while milk (cow and buffalo) from which ghee is prepared contains no lead. Lead in human milk varies very much—from nil to 0·17 mg. per kilo. Fatty foods are more liable to contamination by lead.

5. Food materials when cooked by Indian methods of cooking yield lead figures different from those of the original raw materials—due to addition of accessory food materials such as ghee, salt, spices, etc. in the process of cooking.

6. About 160 samples of common Indian foods, beverages and accessory food materials have been analysed and the result has been classified into nine different groups.

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APPENDIX.

TABLE I.

Lead content of various foodstuffs.

The figures indicate milligrams of lead (as Pb) per kilo or litre as the case may be (or parts per million).

A. *Animal food.*

Meat (goat)	0·45, 0·48, 0·47	Vetki (*Lates calcarifer*)	0·18
Goat's liver	0·18, 0·19, 0·14	Crab (edible portion)	0·24, 0·20
Goat's kidney	0·10		
Mutton	1·35, 1·2, 1·32	Sea fish—	
Mutton liver	2·3, 2·88	Tie (*Plaice*)	0·16, 0·12
Beef	0·30, 0·36, 0·35	Sole (*Cynoglossus lingua*)	*Nil*
Beef liver	0·36, 0·42	Pomfret (*Pampus argenteus*)	*Nil*
Beef kidney	0·2	Halua	*Nil*
Kôi fish (*Anabus testudineus*)	*Nil*	Lady's finger (*Sillago sihama*)	*Nil*
Mâgur fish (*Clarias batrachus*)	0·06	Egg (duck's)	0·3, 0·28
Rûi or rôhit fish (*Labio rohita*)	0·32, 0·54	Egg (hen's)	0·24, 0·25
Shrimp (edible portion)	0·24	Chicken	0·03, *Nil*
Hilsa fish (*Hilsâ ilisha*)	*Nil*	Lobster—fresh-water—(edible portion)	0·08

B. *Vegetables.*

Patôl (*Trichosanthes dioica*)	*Nil*	Baigun or brinjal (*Solanum melongana*)	0·07
Karôla (*Momordica charantia*)	*Nil*	Garlic	0·14
Radish	*Nil*	Cabbage	0·24
Lal kumra or pumpkin (*Cucurbita maxima*)	*Nil*	Cauliflower	0·2
Potato	*Nil*	Sweet potato (*Ipomœa batatus*)	*Nil*
Onion	*Nil*	Green papaya (*Carica papaya*)	*Nil*

TABLE I—*contd.*

B. *Vegetables*—concld.

Lāo or louki or gourd (*Lagenaria vulgaris*).	*Nil*	Sajina danta or drumstick (*Moringa pterigospermum*).	0·08
Shim or ordinary runner bean (*Dolichos lablab*).	0·08	Carrot	0·24, 0·29
Puin sak (*Bassela rubra*)	0·2	Chalkumra (*Benincasa cerifera*)	0·26
Pālong sak or spinach (*Spinacia oleracea*)	0·24	Bhindi or lady's finger (*Hibiscus esculentus*)	*Nil*
Natia sak (*Amarantus gangeticum*)	0·08	Lettuce	*Nil*
Karai sunti or green peas (*Pisum sativum*)	0·12	Celery	*Nil*
Dumur or green figs (*Ficus glomerata*)	0·32	Kanch kala or green plantain (*Musa sapientum*).	0·2
Kalmi sak (*Ipomœa reptans*)	0·28	Thore—core of trunk of *Musa paradisiaca*	0·04
Beet	0·21	Betel leaf	*Nil*
Jhinga or ridge gourd (*Luffa acutangula*)	0·18	Jack-fruit seeds (*Artocarpus integrifolia*)	0·12

C. *Cereals.*

Rice, parboiled	*Nil*	Khesari dhal (*Lathyrus sativus*)	0·16
Rice, sun-dried	*Nil*	Kalai dhal or green gram (*Phaseolus radiatus*).	0·28
Matôr dhal (*Pisum sativum*)	0·12		
Chhola, boot or chana dhal (*Cicer arietinum*).	0·24	Wheat flour—	
Moog dhal (*Phaseolus mungo* var. *aurea*)	0·16	Hand-ground	0·16
Masoor dhal (*Lens esculentum*)	0·12	Milled	0·28
Arhar dhal (*Cajanus indicus*)	0·04, 0·06	Soya bean (*Glycine hispida*)	*Nil*

D. *Fresh fruits.*

Mango (*Mangifera indica*)—		Bael fruit pulp (*Ægle marmelos*)	*Nil*
Langra	0·06	Cucumber (*Cucumis sativus*) with skin	0·2
Fazli	0·08	Khôrbuja (musk melon) (*Cucumis melo*)	*Nil*
Orange	0·32, 0·56	Lichi (*Lichi sinensis*)	*Nil*

TABLE I—*contd.*

D. *Fresh fruits*—concld.

Jam or jamun or black berry (*Eugenia jambolana*).	*Nil*	Golap jam (*Eugenia jambos*) . . .	0·08
Guava (*Psidium guyava*) . . .	0·24	Loquat (*Eriobotrya japonica*) . . .	0·04
Ripe papaya (*Carica papaya*) . .	*Nil*	Coco-nut (*Cocos nucifera*) . . .	*Nil*
Jack-fruit (*Artocarpus integrifolia*) . .	0·13	Tal sansh or palmstone pulp (*Borassus flabellifomis*).	*Nil*
Grape	*Nil*	Sankh aloo (a tuber) (*Pachyrhizus angulatus*).	0·05
Plantain or banana—Martaban variety (*Musa paradisiaca*).	*Nil*	Amra or hog plum (*Spondias dulcis*) .	*Nil*
Apple (imported)	0·3	Tomato	0·28

E. *Dried fruits* (*mostly imported*).

Apricot	0·24	Dates (*Phœnix sylvestris*) . . .	0·04
Pistachio (*Pistacia vera*) . . .	0·3	Raisins	*Nil*
Almond (*Prunas amygdalis*) . . .	0·56	Dried grapes	*Nil*
Walnut	*Nil*	Ground nut (*Arachis hypogea*) . .	*Nil*

F. *Spices, condiments and miscellaneous.*

Ginger	0·08	Cummin seeds	*Nil*
Red pepper (*Capsicum annum*) . .	*Nil*	Jowan or ajowan (*Carrum curvi*) . .	0·07
Turmeric (*Curcuma longa*) . . .	0·4	Cinnamon	0·03
Betel nut (*Areca catechu*) . . .	0·08	Common salt—	
Aniseed (*Fœniculum vulgare*) . . .	*Nil*	Karkach (Aden salt) . .	0·42, 0·45
Coriander seed	0·06	Saindhab (rock salt) . .	0·25, 0·26
Mustard seed (*Brassica nigra*) . .	0·4	Liverpool salt	0·2
Black pepper	0·22	Bengal salt	0·4
Cloves	0·15	Sugar (country made)	0·004
Curry powder	0·32	Gur (from sugarcane)	*Nil*
Cardamom	0·23	Chutney (mango slice) . . .	0·18

TABLE I—*concld.*

G. *Fatty foods.*

Milk (human)—		Teel oil (sesame oil)	0·6
Bengali	0·16, *Nil*, *Nil*, 0·14, 0·17	Sialkanta oil (*Argemone mexicana*—used as an adulterant of mustard oil).	0·33
Punjabi	0·01	Ghee—buffalo (tinned)	0·56
European	0·04	Ghee—home-made from cow's milk	0·10
Milk (cow)	*Nil*, *Nil*	Butter (tinned)	0·48
Milk (buffalo)	*Nil*	Cheese (imported)	*Nil*
Rape oil (fixed mustard oil)	0·45	Khôa (a milk product)	0·024
Coco-nut oil	1·20, 1·32		

H. *Beverages.*

Tea (packed in lead foil)	2·4	Country spirit	0·026
Tea (packed in tins)	1·8	Whisky (country made)	0·027
Brandy (country made)	0·05, 0·06	Aerated waters—	
Rum (country made—two different brands).	0·024, 0·057	Lemonade	0·004
Dry gin (country made)	*Nil*	Soda	0·004

TABLE II.

Soil and water. Milligram of Pb per kilo or litre (or p.p.m.).

Tap-water (Calcutta water-supply)	0·002	Tank-water (College Sq. tank)	0·001
Tube-well water (250 feet deep, Calcutta)	0·0015	Soil (Calcutta)	0·06
Tube-well water (250 feet deep outside Calcutta).	0·0015	Soil (from a depth of 15 feet)	0·04

TABLE III.

Lead content of cooked foods. Most of them were cooked by Indian method of cooking. The figures indicate milligrams of lead (as Pb) per kilo (or p.p.m.).

White bread (from imported white flour)	0·2	Potato and patôl curry	0·32
Rôti, chapatti (brown flour)	0·18	Masoor dhal (thick soup)	0·18
Luchi, puree (white flour)	0·30	Jilabi (with added yellow pigment)	0·20
Cooked rice (cooked in earthenware handi).	*Nil*	Rabri	*Nil*
Cooked rice (cooked in aluminium vessel)	*Nil*	Rasogolla	0·03
Hilsa fish curry	0·1	Sandesh	*Nil*
Potato and rûi fish curry	0·38	Biscuit (country made)	0·20
Mutton korma	1·34	Biscuit (imported)	0·03

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METALLIC CONTAMINATION OF FOODSTUFFS.

Part III.

THE EFFECT OF CONTINUED ADMINISTRATION OF TIN FROM TINNED BRASS VESSELS ON GROWTH—THE EXCRETION AND ABSORPTION OF TIN IN THE RAT.

BY

N. C. DATTA,

Lady Tata Memorial Scholar.

(From the Grant Medical College, Bombay, and Department of Biochemistry, Indian Institute of Science, Bangalore.)

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Traces of tin have been reported to be present in the human and cow's milk as also in the ash of human blood. It is abundant in the brain, spleen and thyroid. Misk (1933) found tin varying from 5·9 mg. per kg. in the human brain to 137 mg. in the liver and spleen. Tin appears to be a normal constituent of the living organism but its exact physiological function in the body has not been properly understood.

White (1880) was the first to study the fate of tin in the body. He found that the urine collected during 12 hours after the intravenous injection of tin salts contained appreciable quantities of the metal. The different organs, such as muscles, liver and brain, were found to contain tin about four or five days after the injection. Ungar and Bodlander (1887) reported that tin given subcutaneously either to a dog or a rabbit was found to be eliminated both in the urine and in the fæces; the quantities in the urine were far greater than in the fæces. The liver contained tin in considerable quantities but the amount in the muscles was, however, very small. Salant, Rieger and Treuthardt (1914) working on the toxicity of metals found that following subcutaneous injection of the tartrate, tin was chiefly eliminated by the gastro-intestinal tract, the kidney playing only a subnormal rôle in the excretion of tin from the body. Tin salts given by mouth to rabbits or dogs showed no absorption from the intestinal canal, only traces of the metal appeared in the urine. Absorption of tin was observed in the case of rats after feeding

them for about four months with tin salts. The authors concluded that continued feeding possibly caused changes in the mucosa of the gastro-intestinal tract favouring absorption of the metal. Salant (1920) reported that on feeding cats with tin as tartrate or chloride, no harmful effect was observed, the excretion being chiefly through the intestine. Flinn and Inouye (1928) observed that in the rats the excretion of tin in the fæces was about 98·5 to 99 per cent of the amount ingested. Schwartz and Clarke (1927) studied the effect of administering tin in the form in which it occurs in canned foods. Diets containing 777 p.p.m. of tin given to guinea-pigs produced no abnormal conditions in the animals. All the animals grew well. Hughes (1935) writing on metallic contamination of foods suggested that 'some definite opinion as to the reasonable safety limit for the unavoidable occurrence of traces of metals in food is desirable'. The question of copper, nickel and tin which are less avoidable and much less harmful, is a matter requiring some definite and unbiased pronouncement. This is a matter of importance not only to the food manufacturer but also to the public as users in the household, of metal cooking utensils.

Tinned vessels are extensively used in India, especially in the South, where foodstuffs highly acidic and containing salts are cooked and stored in such vessels for various lengths of time with the result that varying quantities of the metal are being constantly taken in along with the food. The quantity of tin thus entering the system, though small in a single dose, may yet become significant if taken regularly, so that it is not improbable that the cumulative effect of the metal is already being felt. In view of the pressing need for further information on the subject the present metabolic studies of tin in rats were undertaken in order to study the mode of excretion of tin from the body, the extent to which the metal is deposited in different organs, the change, if any, brought about by the metal accumulated on the cell structure of the tissues and, finally, the effect on the growth and well-being of the animals, as a result of continued feeding of foodstuffs containing tin derived from the vessels.

Experimental.

Investigation on the effect of food prepared in tinned vessels on the growth of rats.

In a previous communication (Datta, 1934), it has been shown that the tinned vessels and utensils which are largely used in India for cooking and storage of foodstuffs often contain lead owing to the fact that lead in varying quantities is mixed with the tin used for tinning such vessels. It was further shown that foodstuffs prepared in those vessels containing tin adversely affect the growth of animals. Whether the adverse effect on the growth of animals was due to lead alone or due to a combined action of lead and tin, is still a problem that remains to be solved. Young rats weighing about 40 g. to 45 g. were divided into three groups. The first group of rats was given rice, dhal and vegetables (which represent an average Indian diet) cooked and stored in a brass vessel tinned with pure tin; the second group of animals was given the same diet but prepared and stored in a brass vessel tinned

PLATE X.

Rats getting food prepared in :

(1) Brass vessel tinned with alloy of lead and tin.
(2) Brass vessel tinned with pure tin.
(3) Glass vessel.

with an alloy of 50 per cent lead and 50 per cent tin. The third set of animals was kept on the same diet prepared in a glass vessel for control. The feeding experiment was carried on for five months, the results being shown in Table I:—

TABLE I.

Average growth of a group of rats maintained on food prepared in a brass vessel tinned with pure tin as compared with a second group getting similar food prepared in a brass vessel tinned with an alloy of lead and tin.

Vessel used.	Number of rats employed.	Days under experiment.	Average weight in grammes.		
			Initial.	Final.	Increase.
Glass	12	142	45	130	85
Brass tinned with pure tin	15	142	48	117	69
Brass tinned with alloy of lead and tin.	10	142	50	105	55

The result given in Table I will show that the rats fed on food prepared in brass vessel *tinned with pure tin* was about 15 to 20 per cent lower than those of the control. The animals otherwise appeared quite normal and healthy and no abnormality or poisoning effect was observed in any case. The animals of the third group appeared less healthy than the animals of the other groups and the average growth of this group of rats was about 35 per cent lower than the control.

Metabolic experiment on the excretion of tin in rats.

Young rats weighing about 60 g. to 70 g. from stock colony were kept in separate glass cages designed for the purpose. The top of the cage was left open, the bottom was made in the form of a screen with thin glass rods inserted in a wooden frame. The cages were placed on large glass funnels, below the stem of which was placed a glass bottle to collect the urine. The fæces were retained on a perforated porcelain disc kept within the funnel. The urine and fæces were collected daily. The fæces were dried and kept in a bottle, the urine was collected and charred with sulphuric acid in a Kjeldahl flask. The funnel was washed every day with hot distilled water and the washings were added to the urinary material. Estimation of tin was carried out once in 15 days.

Most of the experiments on the excretion of tin referred to in the literature was carried out either by subcutaneous or intravenous injection of inorganic tin salts

to the animals or by adding the salts to the diet of the animals. There are indications that the manner in which the inorganic elements are used in the body depends on the form in which they are presented to the animal. In the present investigation, excretion of the metal was followed in rats with the types of acid foods that are generally cooked or stored in the tinned vessels, especially in South India.

In the first series of the experiment, the animals were given wheat *chapatti* and *acid curd* prepared and stored in tinned brass vessels. Three rats were kept in separate metabolic cages. Each rat was given 10 c.c. of curd before giving chapatti and care was taken to see that they took all the curd. Any curd that was left over in the vessel was washed and kept separately for the estimation of tin and the value was deducted from the quantity of tin present in the 10 c.c. of curd given to each rat. An aliquot portion of 10 c.c. of curd was kept separately each day for the estimation of quantity of tin present in the given diet. The experiment was conducted for 35 days. The collection of urine and fæces was stopped two days after the last meal containing tin was given. For the estimation of tin, the organic matter was destroyed by digestion with sulphuric and nitric acid. The tin sulphide was then obtained in the usual way by precipitation with hydrogen sulphide. The precipitate of tin sulphide was dissolved in hydrochloric acid and a little potassium chlorate and reduced to stannous condition by the method of Clarke (1931) in an atmosphere of carbon dioxide. A simple apparatus similar to that described by Evans (1927) was used for the purpose. Finally, a known amount of N/100 iodine was added and the excess of iodine was determined by titration with thiosulphate solution. From the amount of iodine used up, the quantity of tin in the given sample was calculated.

TABLE II.

Excretion of tin in rat kept on wheat chapatti and 10 *c.c. of curd daily. The curd was prepared and stored in tinned brass vessel.*

Rat number.	Tin intake, mg.	Total tin excretion in mg. in		Per cent of total tin excreted in		Per cent of tin recovered.
		Fæces.	Urine.	Fæces.	Urine.	
1	6·12	5·51	0·38	90·0	6·2	96·2
2	5·84	5·35	0·33	91·6	5·6	97·2
3	5·37	4·81	0·30	89·5	5·5	95·1

It can be seen from the result given in Table II that 90 per cent of tin taken along with the acid curd is excreted in the fæces and about 5·7 per cent in the urine. The total excretion in urine and fæces is on an average about 96 per cent. After making allowance for slight experimental error, there remains about 3 per cent of tin to be accounted for.

In the second series of experiment, six young rats weighing about 70 g. to 80 g. were divided into two groups and were kept in separate metabolic cages. The animals of the first group were kept on wheat chapatti, 10 c.c. of milk and 10 c.c. of rasam prepared in tinned vessel. The animals of the second group were given wheat chapatti, 10 c.c. of whey and 10 c.c. of rasam. The diet of both the group contained the same amount of tin but differed only in their protein content. Rasam is a preparation most common in South India and contains water extract of green pulses, tamarind, salt and various spices. It is a liquid preparation, highly acidic and cooked in tinned vessels. Rasam was given to the animals mixed with the milk, so that the rats may not refuse to take it.

The collection of urine and fæces and the sampling of food for the estimation of tin were carried out as in the first series of experiment.

TABLE III.

The excretion of tin in rats maintained on wheat chapatti supplemented with 10 c.c. of rasam (prepared in tinned vessel) mixed with milk in group I, and 10 c.c. of rasam mixed with whey in those of group II.

Rat number.	Days under experiment.	Total tin intake, mg.	TOTAL TIN EXCRETED IN MG. IN		PER CENT OF TIN EXCRETED IN		Per cent of tin recovered
			Fæces.	Urine.	Fæces.	Urine.	
			GROUP I.				
1	150	100·57	86·08	5·43	85·5	5·3	90·9
2	150	91·33	79·79	4·65	87·3	5·0	92·4
3	150	73·64	64·78	3·14	87·9	4·2	92·2
			GROUP II.				
1	128	53·96	46·16	3·01	85·5	5·5	91·0
2	128	68·96	56·82	2·96	82·3	4·2	86·6
3	128	67·56	49·29	3·61	72·9	5·3	78·3

The results in Table III show that in the animals of group I, about 91·9 per cent of tin present in the diet supplied is excreted in the fæces and urine ; the amount in the fæces being about 86·8 per cent. In the animals of group II, the rate of excretion of tin appears to be slower. The amount of tin in the urine is the same

in both the cases and is about 4·9 per cent. The slight difference in the rate of excretion of tin in fæces between the two groups may be due to the difference in the protein content of their diet. It is known that proteins form a complex with the heavy metals and may therefore facilitate their excretion.

TABLE III-*a*.

The rate of excretion of tin in rat No. 3 (group I) during the whole course of the experiment.

Number of days.	Tin intake, mg.	Tin excreted in mg. in		Total excretion of tin in urine and fæces, mg.
		Fæces.	Urine.	
15	9·46	6·59	0·11	6·70
15	8·09	8·20	0·11	8·61
15	8·70	8·41	0·36	8·77
15	6·90	3·41	0·44	3·85
15	5·70	3·40	0·32	5·72
15	9·25	13·47	0·40	13·87
15	8·81	7·90	0·29	8·19
15	6·99	6·64	0·30	6·94
15	3·79	3·97	0·30	4·27
15	5·95	2·79	0·21	3·00
150	73·61	64·78	3·14	67·92

Per cent of tin excreted in fæces = 87·9
Per cent of tin excreted in urine = 4·2

Per cent recovered .. 92·2

TABLE III-*b*.

The rate of excretion of tin in rat No. 2 (group II) during the whole course of the experiment.

Number of days.	Tin intake, mg.	Tin excreted in mg. in		Total excretion of tin in urine and fæces, mg.
		Fæces.	Urine.	
8	4·84	2·59	0·21	2·80
15	8·07	6·70	0·40	7·10
15	9·25	10·08	0·53	10·61
15	11·25	7·93	0·41	8·37
15	10·23	6·55	0·31	6·86
15	9·38	7·51	0·29	7·80
15	6·22	6·49	0·24	6·73
15	3·79	4·45	0·20	4·65
15	5·93	4·51	0·34	4·85
128	68·96	56·82	2·96	59·77

Per cent of tin excreted in fæces = 82·3
Per cent of tin recovered .. = 86·6

From the result of analysis carried out for periods of 15 days as shown in Table III, it is clear that there is a tendency for a slight but definite amount of tin to be retained in the body at every stage. The final result drawn from an average of the values of six rats in Table III shows that about 90 per cent of the metal ingested can be recovered from the excretions. The remaining 10 per cent of the tin is either absorbed and stored in various tissues or retained in the body to be slowly excreted, as indicated by the result in Table IV. Schryver carried out a feeding experiment on himself by taking one grain of tin as tartrate daily for a week and gradually increasing to three grains in the third week. He observed a retention of two grains of tin during the third week which was however slowly excreted.

Tin in different tissues.—In order to get an idea as to the amount of tin that may be stored in various tissues, the animals from the groups of rats employed in the previous experiment were killed, the organs were removed and freed from the adhering blood. The liver, kidney, heart and lungs were then analysed for tin content. From the result of analysis it appeared that the liver and kidney are the main storage place for the metal. The accumulation of tin in the liver was not very marked. The concentration of tin in the kidney was found to be considerably greater than in the liver. In the case of two rats, the kidney weighing 1·28 g. and 1·52 g. were found to contain 1·43 mg. and 1·06 mg. of tin, respectively. The values appeared to be surprisingly high and are therefore being re-investigated. The results indicated the possibility that a maximum value of about 2 per cent of the tin ingested may be stored in the tissues, the rest being present in the intestine or colon in the process of being eliminated.

A preliminary histological examination made on liver and kidney of rats, one from each group, showed no evidence of injury to the organs when sections of these were compared with similar sections from control animals. Re-investigation of the problem from the point of view of storage of tin in different tissues and their effect on the cell structure of these tissues by histological examinations is under progress.

The investigation on the time taken in days for the maximum excretion of tin on oral administration in a single dose.

In order to find out the duration to which tin is retained in the body in the process of its elimination in the urine and fæces, tin as stannous chloride was given to the rats mixed with milk and the excretion of the metal was followed. Tin was estimated in the sample of fæces collected daily. Tin was analysed in the total quantity of urine collected during six days. The result in Table IV gives the average of tin intake and the excretion of the metal in the urine and fæces of three rats employed in the experiment :—

TABLE IV.

The time taken in days for complete excretion of tin when 13·65 *mg. of tin as stannous chloride was given to rats mixed with milk, in a single meal.*

Number of rats used.	Tin intake, mg.	Number of days.	Tin excreted in mg. in fæces.	Tin in mg. in urine.
3	13·65	1st	1·41	..
		2nd	4·54	..
		3rd	3·21	..
		4th	1·23	..
		5th	1·12	..
		6th	0·86	0·61
		TOTAL ..	12·37	0·61

Total excretion of tin .. 12·98 mg.
Amount unrecovered .. 0·67 mg.

The data presented in Table IV show that a quantity of stannous chloride equivalent to 13·65 mg. of tin taken along with milk in a day required more than six days for its excretion. About 75 per cent of the metal was excreted in the fæces during the first three days, the rest appearing only slowly after this. The result of the experiment clearly indicates that a quantity of tin after oral administration along with other food though mostly excreted in the fæces yet the process being

slow, definite but decreasing quantities of the metal are invariably retained in the body for a fairly long period before most of it is excreted. The duration of such retention of the metal and also the quantity may still be greater if tin is constantly fed for a fairly long time.

Distribution of tin in different parts of the alimentary canal in the process of excretion in the fæces.

The metal taken along with the food is mostly excreted in the fæces but as the process is slow, various quantities of the metal are retained in its path before most of it is excreted. Investigation was therefore carried out to find out the distribution of tin in different parts of the alimentary canal at different intervals of time. Four rats were employed in the experiment and were given the same quantity of tin as stannous chloride along with the milk. The actual intake of tin was determined by deducting the value of tin in the milk left over from the original quantity added to the milk. The fæces were collected at intervals of 24 hours. One animal was killed after every 24 hours and the stomach, duodenum and the intestine were removed and analysed for tin. The results are included in Table V :—

TABLE V.

Distribution of tin in fæces and also in different parts of the alimentary canal at different intervals of time after oral administration.

	Rat No. 1	Rat No. 2	Rat No. 3	Rat No. 4
Tin intake, mg. :—	20·23	17·65	20·06	21·14
Distribution after :—	24 hours.	24 hours.	48 hours.	96 hours.
		In mg.		
Stomach ..	1·25	1·60	1·05	0·21
Duodenum ..	1·65	1·85	0·91	0·75
Intestine ..	8·62	8·49	5·30	3·82
Fæces	7·34	4·10	11·32	14·50
TOTAL ..	18·86	16·04	18·58	19·28
Amount of tin unrecovered.	1·37	1·61	1·48	1·86

A portion of the tin unrecovered could be accounted for by analysing the urine collected during the period and also the tin content of the kidney.

The results in Table V show that on oral administration of tin about 30 per cent of the metal appears in the fæces during 24 hours, the remaining 70 per cent remaining distributed in the different parts of the alimentary canal. As the metal descends down, the quantity in fæces gradually increases. As in the case of rat No. 4, about 75 per cent of the tin was excreted in the fæces, during 96 hours, thus leaving a quantity equivalent to about 15 per cent in the intestine and much less quantities in stomach and duodenum. The quantities of metal still retained may take two to three days more for their complete excretion.

Discussion.

The results of the present investigation carried out as far as possible with scrupulous care show that tin in quantities in which it occurs in food prepared in tinned brass vessels when fed to animals produces a slight retarding effect on the growth of animals. The growth of rats was found to be about 15 per cent lower than the control, when food prepared in tinned vessel was fed for 142 days. Schwaibold and Fischer (1933) carried out investigations with tadpoles in which they kept the animals in water containing 2 mg. of tin as stannous chloride for 1 litre of water and observed that, though the animals appeared quite normal, the growth of tadpoles thus treated was 88·2 per cent of the control.

The result of the metabolic experiment, allowing for experimental error due to collection of fæces and urine and also due to sampling of food, and error inevitable in experiments like this, reveals that about 85 per cent of the tin ingested along with the food is excreted in the fæces, in case the experiment is concluded within a day or two after the last meal containing tin is given. A part equivalent to about 8 to 10 per cent is retained in the different parts of the alimentary canal for a fairly long period and is only slowly excreted in the fæces. The rate of excretion of the metal depends on the nature and constituents of the diet. With a diet containing milk, the excretion of the metal appears to be quicker possibly due to the formation of protein complex which is easily excreted. The elimination of metal takes place mostly through the gastro-intestinal tract. The quantity of metal in the urine was found to be very small showing that the absorption of the metal from the intestine is very slow. The accumulation of tin in the liver is not very marked, but the concentration of tin in the kidney was found to be much greater than in the liver. A preliminary histological examination revealed no injury to the cell structure of such tissues as liver and kidney.

Summary.

1. The growth of rats fed with food prepared in tinned brass vessels was found to be about 15 per cent lower than those of the control getting the same food prepared in glass vessels.

2. The result of metabolic experiment on the excretion of tin in rats indicated that about 90 to 95 per cent of the metal was excreted in the fæces. A part of

it is, however, retained in the body for a fairly long time and is only slowly excreted. It has been observed that a quantity of tin as stannous chloride given in a single dose along with milk requires about seven days for its complete elimination in the fæces. During this period of retention of tin in the body, the metal remained distributed in different parts of the alimentary canal. The quantity of metal in the urine was found to be very moderate.

3. The accumulation of tin in the liver is not very marked. The concentration of tin in the kidney was found to be much higher than in the liver. A preliminary histological examination of the liver and kidney of these rats did not reveal any injury to the cell structure of the tissues.

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CHEMICAL ASSAY OF 'RASAUT' AND 'HING' FROM THE PUNJAB MARKET.

BY

K. S. GREWAL, M.B., B.S. (Pb.), Ph.D. (Cantab.),

AND

B. D. KOCHHAR, M.Sc.

(From the Department of Pharmacology, King Edward Medical College, Lahore.)

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To obtain an idea of the purity of indigenous drugs as sold in the market, two well-known indigenous drugs—'Rasaut' and 'Hing'—were chemically assayed.

(A) Rasaut.

Rasaut is sold in the bazaar as a brown almost solid extract wrapped in leaves. It is a common practice to dissolve it in water; the insoluble part is removed and the solution is again evaporated to dryness. This is in itself a proof that the extract is adulterated with insoluble substances but the public has learnt to regard it as a normal state of affairs.

Rasaut is used as a local application in affections of eyes and eyelids. Internally it is used as a bitter and a diaphoretic in intermittent fevers. It is used in Hindu medicine from a very remote period and is described in all the Hindu books on materia medica. In the 'Pharmacographia' by Flückiger and Hanbury (1879), the following account is given of the antiquity of its use:—

'The medical practitioners of ancient Greece and Italy made use of a substance called Lycium, of which the best kind was brought from India. It was regarded as a remedy of great value in restraining inflammatory and other discharges; but of all the uses to which it was applied, the most important was the treatment of various forms of ophthalmic inflammations.'

Innumerable conjectures were put forth during at least three centuries as to the origin and nature of Lycium and especially of that highly esteemed kind that was brought from India.

In the year 1833, Rayle communicated to the Linnaon Society of London a paper showing that the Indian Lycium of the ancients was identical with an extract prepared from the wood or root of several species of *Berberis* growing in Northern India and that this extract well known in the bazaars as Rusot or Rasot was in common use among the natives in various forms of eye diseases (Flückiger and Hanbury, *loc. cit.*).

Rasaut has been used by a number of practitioners of Western medicine in the last century and is well spoken of as an antiperiodic and antipyretic.

Rasaut sold in the Punjab comes mostly from Jammu and Kashmir State. The method of its preparation on a commercial scale as practised was described by Loregrone (1914).

We have examined fifteen samples of Rasaut bought in the local market and from Amritsar. It contains an active principle, an alkaloid—berberine. The results are set forth in Table I :—

TABLE I.

Analysis of Rasaut samples.

Sample number.	Moisture, per cent.	Insoluble and foreign matter, per cent.	Ash, per cent.	Alkaloid, per cent.	Soluble Rasaut, per cent.	Alkaloid in pure Rasaut, per cent.
1	14·3	15·1	7·1	0·03	70·6	0·04
2	23·3	18·3	17·0	0·06	58·4	0·10
3	11·5	14·0	9·0	*Nil*	75·5	Absent.
4	14·3	14·1	7·4	1·67	71·6	2·33
5	10·8	13·5	7·5	*Nil*	76·6	Absent.
6	9·8	27·5	18·0	*Nil*	62·7	Absent.
7	15·3	9·1	7·7	2·3	75·6	3·04
8	14·5	12·7	8·0	*Nil*	72·8	Absent.
9	16·2	11·9	6·6	0·20	71·9	2 79
10	15·7	20·2	14·5	0·11	64·1	1·87
11	11·5	15·1	10·0	Traces	73·4	Traces.

TABLE I—*concld.*

Sample number.	Moisture, per cent.	Insoluble and foreign matter, per cent.	Ash, per cent.	Alkaloid, per cent.	Soluble Rasaut, per cent.	Alkaloid in pure Rasaut, per cent.
12	12·9	26·8	20·4	0·16	60·3	2·65
13	9·5	22·1	19·0	2·65	68·4	3·86
14	10·1	18·1	13·8	1·2	71·8	1·67
15	*Nil*	17·8	3·34	3·5	82·2	4·26

Two more drugs are recommended in India for eye diseases—Mamira (*Coptis tita*) and Mamiri (*Thalictrum javanicum*). It is a curious fact that both these drugs contain berberine. *Coptis tita* was shown to contain berberine by other workers. *Thalictrum javanicum* has been shown by us to contain berberine. We isolated as much as 1·3 per cent of berberine from its rhizome.

(B) HING.

Hing is a gum resin consisting of blackish brown, originally translucent, brittle masses of extreme fœtid, alliaceous odour. This is used in medicine and also as a household remedy and in cooking. It is said to be derived from a plant—*Ferula alliacea*—found in Eastern Persia in the neighbourhood of Djendack and Yezd and in Khorassan near Seharud, Nischapur, Meshed, Dehrachtindjan and Kerman (Watts, 1890).

Another plant *Ferula fœtida*, which yields a similar gum resin called Hingra in Bombay, grows in whole of Southern Turkistan as far north as the river Syrdarja, in Eastern Persia, in Khorassan, and the neighbouring parts of Afghanistan near Herat. It has been collected further north in Central Asia between the Caspian and Sea of Aral. This is asafœtida of the B. P.

It has been estimated that on an average about 6,000 cwt. of Hing valued at Rs. 2,16,300 are brought annually by Afghan merchants and sold to the Frontier towns (Chopra, 1933*a*).

The B. P. lays down that asafœtida should contain not less than 50 per cent alcohol-soluble resin and the ash content should not be more than 15 per cent.

Ten samples of Hing, which were procured from reputable sellers of indigenous drugs, were examined and compared with two samples of asafœtida procured from well-known dispensing chemists. One of the dispensing chemists was found to be selling Hing for asafœtida (B. P.). The sample of Hing, which he was selling, was grossly adulterated. It had 26·3 per cent of alcohol-soluble resin, 33·07 per cent of ash and the adulterant was chalk. Out of the ten samples of Hing, only one contained alcohol-soluble resin over 50 per cent and ash within accepted limits. Eight of the samples showed alcohol-soluble resin below 30 per cent and ash

contents above 25 per cent for those adulterated with gypsum and below 10 per cent for those adulterated with resin.

The result of analysis of ten samples of Hing and two samples of asafœtida (B. P.) is given in Table II :—

TABLE II.

Analysis of Hing and asafœtida samples.

Sample number.	Solubility in alcohol, per cent.	Ash contents, per cent.	Adulterant.
1	21·034	11·00	Gypsum and rosin.
2	15·80	47·00	Gypsum.
3	14·508	6·250	Rosin.
4	18·186	5·07	Rosin.
5	58·520	9·09	Pure.
6	21·924	35·615	Chalk.
7	29·97	37·04	Chalk.
8	16·53	4·45	Rosin.
9	43·26	25·227	Chalk.
10	25·29	46·1	Chalk.
11	26·332	33·07	Chalk.
12	76·82	3·15	Pure.

The results were discussed by us with the leading Vaids and one of them has kindly sent three samples which were regarded as extremely pure by him for analysis. The result of analysis is shown in Table III :—

TABLE III.

Analysis of three samples of Hing sent by a Vaid.

Sample number.	Solubility in alcohol, per cent.	Ash contents, per cent.	Adulterant.
1	57·53	12·15	*Nil.*
2	28·27	5·75	Chalk and rosin.
3	27·98	2·535	Rosin.

Discussion.

Samples of Rasaut and Hing, which were collected from reputed dealers of indigenous drugs, on examination showed gross adulteration. The samples were collected at random and could be taken as representatives of those sold in the market. It is, therefore, concluded that there is a great deal of adulteration going on. The drugs not being particularly expensive or difficult to procure, it is presumed that such adulteration may also be going on with other drugs of similar nature.

The use of indigenous drugs is increasing and with the increasing patronage of the Government that the indigenous systems are acquiring in some provinces, the use of such drugs will increase rapidly. It is time, therefore, that the question of laying down standards of purity for such drugs be taken up.

For drugs used in scientific medicine, the Pharmacopœia lays down the source of a drug, its physical and chemical properties, and tests of purity but such exact information is not procurable for indigenous drugs. For example, Brahmi is commonly used in the indigenous medicine as a tonic for memory. There are at least two different plants which are sold under the name of Brahmi. In the Punjab *Hydrocotyle asiatica* is sold as Brahmi but Bengal Vaids use *Herpestis monniera.* Chopra (1933*b*) in his book on indigenous drugs has described *Herpestis monniera* as Brahmi. Likewise there are other drugs such as Rasna. In the Punjab leaves of doubtful botanical source are sold as Rasna and in Bengal roots of *Vanda roxburghii* and of *Acampe papillose* are indiscriminately used as Rasna (Kirtikar and Basu, 1918). The problem is fairly serious because in Amritsar drug market as many as fifty drugs out of about 400 that the Ayurvedic pharmacies are handling, are disputed. Drug under a common name is sold differently at different pharmacies.

The investigation of any indigenous drug to be completed should include in its study macroscopic and microscopic characters and means of recognition of the crude drug, general adulterants and their mode of detection, the source of the drug and the variation that it undergoes due to its environments. It is further necessary to find the general properties of the crude drug and its active principle which is the source of its properties. To make it available in therapeutics, it is necessary to know the history of the drug, the preparations in which it forms a part, the process of preparing it, the dose of the crude drug and its preparation. Last of all, it is necessary to know the pharmacology of it. It is a difficult task and it is hoped that the problem will receive more extended attention than it receives now.

Summary.

Fifteen samples of Rasaut and equal number of samples of Hing were analysed.

Out of fifteen samples of Rasaut, seven either contained no alkaloid or only traces; in the rest the variation in the alkaloidal contents was from 1·67 to 4·26 per cent.

Thirteen samples of Hing were examined; only two samples contained more than 50 per cent alcohol-soluble resin, the other eleven were grossly adulterated. Two samples of asafœtida were examined. One was a sample of Hing sold as asafœtida and was grossly adulterated.

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A NOTE ON THE CHEMISTRY AND PHARMACOLOGICAL ACTION OF *ENTADA PURSÆTHA* DC. (*E. SCANDENS* BENTH.).

BY

BREVET-COLONEL R. N. CHOPRA, C.I.E., M.A., M.D., SC.D. (Cantab.), F.R.C.P. (Lond.), I.M.S. (*Retd.*),

J. C. GUPTA, M.B. (Cal.),

CAPTAIN G. S. CHOPRA, M.B., B.S., A.I.R.O.,

AND

B. K. GHOSH, M.SC.

(*From the Department of Pharmacology and Chemistry, School of Tropical Medicine, Calcutta.*)

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INTRODUCTION.

Entada pursætha, a leguminous climbing shrub, is commonly known in English as Giant Rattle, Lady Nut, Mackay Bean, Nicker Bean ; in Hindi, Gila ; in Bengali, Gilagach, Pangra ; in Santali, Bidhanta ; in Uriya, Geredi, Girdi ; in Telegu and Tamil, Gilatege, Irikki ; and in Kanarese, Doddaganpi, Hallekaijiballa, Doddakampi. In Bombay Presidency it is known as Gorambi, Garbi, Gardal, Gharbi, Pilapapra ; in the Punjab, Kastori Kaman ; in Bhutan, Kulhokrik, Taktokhyam. The plant grows in the central and eastern Himalayas at an altitude of 4,000 feet ; in the damp forests of Eastern Bengal, Bihar and Orissa ; in the forest regions of the Eastern and Western Ghâts, and in the hilly forest tracts of the Northern Circars and the Deccan. The pods of this plant are several feet long and about 3 to 5 inches wide. The seeds, largely employed for crimping linen in Bengal and the United Provinces, are about 3 to 4 centimetres in diameter and discoid in shape. The outer covering of the seed, about $\frac{1}{16}$ of an inch thick, is tough, horny,

chestnut coloured and shining in appearance. The white kernels of the seeds are eaten by poor people who first soak them in water and subsequently roast them. If taken otherwise, these seeds produce toxic symptoms such as vomiting and drowsiness. Mentions have been made regarding its medicinal uses. Some hill tribes of India make use of these seeds in the same way as soap to wash their hair. A paste prepared from the seeds is used locally for inflammatory swellings of the glands. Poultice made from the kernel applied locally, is believed to relieve colicky abdominal pains. The seeds are also used as a fish poison in certain parts of India, South Africa and the Philippine Islands.

Rosenthaler (1903) isolated two saponins, saponins A and B, from the kernels of the seeds. Bacon (1906) obtained 7 to 10 per cent of a saponin as white amorphous powder from the bark. Bacon and Marshall (1906) found this saponin to be highly toxic to rabbits and guinea-pigs, and also observed its powerful hæmolytic properties. The saponin has also a destructive action on the amœbæ and some flagellates found in tap-water and in the water of stagnant ponds. It was noted, however, that air bacteria grew abundantly in a solution of the crude saponin.

The object of the present investigation was to isolate the pharmacologically active constituents of the seeds and to study their pharmacological actions.

Chemistry.—For a preliminary examination of the constituents, 200 grammes of the air-dried powdered seed kernels were first extracted in a Soxhlet apparatus with petroleum ether. The extract amounted to 8·2 per cent of the powder and consisted mainly of a clear yellow fatty oil. The residue was next extracted with benzene. The extract amounted to only 0·25 per cent of the powder and contained a very small amount of the colouring matters and fat. It was next extracted with chloroform which gave a residue of 1·5 per cent containing some colouring matters, resins and some sterols. The residual powder was then refluxed repeatedly with 85 per cent alcohol. The alcoholic extract, on removal of alcohol, gave a hygroscopic brown mass which frothed strongly in aqueous solution and showed the presence of saponins. Besides the saponins, no other constituent of any pharmacological interest was isolated.

For the isolation and purification of the saponins, about two kilos of the air-dried seed kernels were coarsely powdered and extracted repeatedly with rectified spirit until the saponins were exhausted. The alcohol was recovered and the brown residue dissolved in a little water and de-fatted with petroleum ether. The aqueous solution was then evaporated to dryness *in vacuo*. The residue was extracted repeatedly with boiling alcohol and the hot extracts filtered. On cooling the filtrate, a portion of the saponins was precipitated. A further portion of the saponin was obtained by precipitation with ether. The total yield of saponins amounted to about 5·5 per cent of the kernels. The brown-coloured crude saponins were purified by fractional precipitation with ether until colourless amorphous substances were obtained.

In many of their chemical properties, the two saponins thus obtained were found to be identical. Both of them were white amorphous substances with a

sharp taste, slightly acid to litmus, soluble in water and alcohol, but insoluble in ether, chloroform, benzene and petroleum ether. They were precipitated by basic lead acetate. With concentrated sulphuric acid, they gave a brown coloration and with dilute HCl they suffered hydrolysis. The ash content was negligible. Both the saponins have identical pharmacological actions and the following description refers to both.

Pharmacological actions.

I. *Local action.*—A 1 per cent solution of these saponins applied locally was found not to produce any inflammation of the intact skin; subcutaneous and intramuscular injections produced inflammatory reactions at the sites of injection. They, however, produced inflammation of the mucous surfaces when applied locally; thus, instilled into the conjunctival sac of the rabbit, they produced redness and smarting.

II. *Toxicity.*—The saponins were found equitoxic as determined on the paramæcia, the larvæ of the anopheline and culicine mosquitoes, the fish, the frogs and the white mice. They were found to be toxic to the paramæcia in concentrations up to 1 in 10,000; higher dilutions proved ineffective. The saponins had no effect on the larvæ of the anopheline and culicine mosquitoes even in concentration as low as 1 in 100. The fish kept in a solution of 1 in 20,000 died within 24 hours. The minimum lethal dose for frogs was found to be 0·1 mg. per g. body-weight and that for mice given intravenously was found to be 0·3 mg. per g. body-weight.

All these animals died within 24 hours of administration of these substances. The main symptoms observed in the smaller animals were salivation, vomiting, marked slowing of respiration, diminution of the reflexes and signs of muscular weakness. In the higher animals the toxic doses produced marked acceleration of the respiration followed by slowing and convulsions; death probably resulted from respiratory failure. Post-mortem examination of the animals killed with the toxic doses revealed congestion of the lungs with dilatation of both the auricles. The gastro-intestinal tract showed congestion throughout. The brain, liver, spleen and the kidneys showed no remarkable change.

III. *Action on the hæmopoietic system.*—A 0·05 per cent solution of both the saponins was prepared to study their effects on the human red blood cells in different concentrations. The results are given in the Table (page 472).

IV. *Action on the cardiovascular system:*

(1) *Action on the blood pressure.*—The saponins when injected intravenously in cats under urethane and chloralose anæsthesia produced a sharp fall of blood pressure. The amount of the fall and its duration, however, depended upon the dose of the saponin injected. With smaller doses such as 1 mg. to 3 mg. per kilo. the fall was sudden but recovery was rapid; with larger doses such as 5 mg. to 10 mg. per kilo the fall was significant and was maintained for a considerable time. After atropine no fall in blood pressure was observed.

TABLE.

Showing the hæmolytic effects of the saponins on the human r.b.c. One per cent suspension of human r.b.c. in normal saline was used.

Number.	R.b.c., c.c.	Saponin solution, c.c.	Normal saline, c.c.	1 hour.	2 hours.	21 hours.	REMARKS.
1	1·0	1·0	..	+++	++++	++++	
2	1·0	0·50	0·50	+++	+++	++++	No. 1 starts hæmolysis almost immediately.
3	1·0	0·25	0·75	—	±	++	
4	1·0	0·10	0·90	—	±	+	
5	1·0	0·05	0·95	-	—	—	

From the above table it appears that this saponin has a strong hæmolytic action on human r.b.c.

Complete hæmolysis ++++
Doubtful ,, ±
No ,, —

(2) *Action on the blood vessels.*—The fall in general arterial pressure was always followed by an increase in the volume of intra-abdominal organs such as the intestines and the kidneys. The re-distribution of blood appears to be directed towards the vessels of the splanchnic area. Perfusion of the vessels of the splanchnic area with 1 in 5,000 dilution of the saponins produced an increase in the flow of the perfusate, showing that these vessels were dilated.

(3) *Action on the heart.*—The intact mammalian heart was depressed by doses as 3 mg. to 5 mg. per kilo body-weight. Cardiometer experiments showed slight dilatation of the organ and the myocardiograph experiments showed slight depression of both the auricles and the ventricles. In the isolated mammalian heart a dilution of 1 in 100,000 of the saponin proved depressant to the myocardium and produced a marked decrease in the amplitude of ventricular contraction.

(4) *Respiratory system.*—The effects of the saponins were studied on the respiratory movements of cats under urethane anæsthesia. With 2 mg. to 4 mg. doses the rate and amplitude of the respiratory movements were increased. With larger doses the respiration became irregular and spasmodic. The administration of toxic doses resulted in respiratory distress, and later emphysema and œdema of the lungs. Lethal doses of the saponins produced complete respiratory paralysis.

(5) *Digestive systems.*—Taken by mouth the solutions of both the saponins had acrid taste and promoted flow of saliva. Administered to a cat orally in 15 c.c.

doses of a 2 per cent solution, they produced salivation, retching, vomiting and diarrhœa within an hour of the administration.

The movements of the small intestine were studied in the chloralosed cats by Jackson's enterograph. The saponins in 3 mg. to 4 mg. doses produced diminution of the tone and subsequently inhibited the intestinal peristaltic movements. Addition of the saponins to the suspended isolated kitten's gut in the Dale's uterine bath produced similar effects but less marked.

(6) *Isolated uterus.*—The drug had also inhibitory effects on the movements of isolated guinea-pig's uteri suspended in the Dale's bath.

Summary and conclusions.

Both the saponins isolated from *E. pursætha* have identical actions and are almost equally toxic. These saponins are much less toxic to paramæcia and non-toxic to mosquito larvæ. The main action is upon the hæmopoietic system where they cause hæmolysis of the red blood cells. A sharp fall of blood pressure was observed in experimental animals after doses of saponins, varying from 0·0005 to 0·002 gramme per kilo body-weight. The fall was associated with an increase in the volume of the intestines, and to a lesser extent those of the kidneys. The fall of blood pressure may partly be the result of the dilatation of the vessels of the splanchnic area and partly due to the depressant effects on the myocardium of the heart. The fall in blood pressure was absent in animals given atropine. The saponins have depressant effects upon the respiratory system and death appears to result from respiratory failure. They have also inhibitory effects on the movements of unstriped muscles of the intestines and the uterus.

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A COMPARATIVE STUDY OF *BŒRHAAVIA DIFFUSA* LINN. AND THE WHITE AND RED FLOWERED 'VARIETIES' OF *TRIANTHEMA PORTULACASTRUM* LINN.

BY

BREVET-COLONEL R. N. CHOPRA, C.I.E., M.A., M.D., SC.D. (Cantab.), F.R.C.P. (Lond.), I.M.S. (*Retd.*),

N. K. CHATTERJEE, M.SC.,

AND

S. GHOSH, D.SC., F.I.C.

(*From the Department of Chemistry and Pharmacology, School of Tropical Medicine, Calcutta.*)

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As has been recently pointed out by Chakravarty (1939-40) there is a good deal of confusion as to the real identity of the Ayurvedic drug Punarnava, particularly Swet Punarnava (also known as Sotaghni in Sanskrit) which is widely used as a specific for dropsy, beri-beri, ascites, etc. In the local markets in Bengal, *Trianthema portulacastrum* Linn.—syn. *T. monogyna* Linn.—(family Ficoideæ) is extensively sold by the herbalists and is prescribed by the Kavirajes as Swet Punarnava. But in almost all the texts on Indian medicinal plants, Punarnava has been identified as *Bœrhaavia diffusa* Linn. (family Nyctaginaceæ). *B. diffusa* Linn. (*B. repens* Linn.) has been described by many as the Punarnava of Ayurveda; they also point out that there are two varieties of this species, one bearing white flowers (Swet Punarnava) and the other bearing red flowers (Rakta Punarnava). Unfortunately, however, none of the species of *Bœrhaavia* has been mentioned in any book on systematic botany to possess white flowers, but they are either pinkish or reddish in colour. *Trianthema portulacastrum* may, therefore, be the real Swet Punarnava as its characters agree well with the Sanskrit description and moreover it is used as such by the Ayurvedic practitioners and is known as the Swet Punarnava.

When one asks for Punarnava, a herbalist in Calcutta will usually supply *Trianthema* instead of *Bœrhaavia*, and samples of Punarnava collected from well-known manufacturing firms have been found on botanical identification to be *T. portulacastrum*.

These interesting findings of Chakravarty have led us to make a comparative chemical and pharmacological investigation of the three plants, *B. diffusa* Linn. and the white and red flowered 'varieties' of *T. portulacastrum* Linn. We are thankful to Mr. Chakravarty for kindly collecting and supplying large quantities of the genuine samples of these plants for our work. They were collected during the months of November and December from areas near about Calcutta and dried in air before they were sent to us.

The chemical examination of the plant Punarnava described as *Bœrhaavia diffusa* was first undertaken by Ghosal (1910) who found the presence of a body alkaloidal in nature. Chopra, Ghosh, Ghosh and De (1923) found the presence of a water-soluble alkaloid, which they designated as Punarnavine, and also fair amounts of potassium nitrate and other potassium salts. Rakshit (1923) also found the presence of an alkaloid in the plant *Bœrhaavia repens* which he designated as Punarnava. Agarwal and Dutt (1934) could not at first isolate any alkaloid from their samples of *B. diffusa* Linn. and found the presence of about 0·5 per cent of a crystalline acid, designated as bœrhaavic acid $C_{10}H_{18}O_3$, about 1 per cent of potassium nitrate and tannins, phlobaphenes, sugars, etc. In a later publication Agarwal and Dutt (1935) describe the isolation of the alkaloid punarnavine in a crystalline state as also its properties and reactions. No systematic chemical investigation appears to have been carried out with the plant *Trianthema portulacastrum* Linn. which is given the Sanskrit name of Swet Punarnava although it is stated to contain very small amounts of a saponin-like glucoside (Dymock, Warden and Hooper, 1890–93).

EXPERIMENTAL.

Preliminary examination.

One hundred and fifty grammes of each of the drugs were taken in a glass Soxhlet and extracted exhaustively with different solvents in succession. The solvents were removed in each case and the residues dried and weighed. The results were as follows :—

1. *Bœrhaavia diffusa.*—Petroleum ether 1·0305 ; ether 0·4855 ; chloroform 0·7695 ; absolute alcohol 4·0460, per cent.

The petroleum ether extract was a yellowish soft waxy mass, consisting of sterols, fats, etc., with a little chlorophyll. The ethereal extract was a dark soft mass, soluble in dilute caustic soda and insoluble in mineral acids, and consisted mainly of organic acids. The chloroform extract was a dark mass giving a faint reaction for alkaloids and consisted mainly of organic acids. The alcoholic extract was a dark mass whose aqueous extract showed a positive ferric chloride reaction and gave a precipitate with Mayer's reagent.

2. *Trianthema portulacastrum* (white flowered).—Petroleum ether 1·1705 ; ether 0·8465 chloroform 1·3275 ; absolute alcohol 7·9900, per cent.

The petroleum ether extract was a yellowish soft waxy mass consisting of sterols, fats, etc. The ethereal extract was a dark soft mass consisting mainly of organic acids. The chloroform extract was a dark mass giving a faint reaction for alkaloids but consisted mainly of organic acids. The alcoholic extract was a dark mass whose aqueous extract showed a negative ferric chloride reaction but gave a precipitate with Mayer's reagent.

3. *Trianthema portulacastrum* (red flowered).—Petroleum ether 1·4925 : ether 0·9458 ; chloroform 1·4075 ; absolute alcohol 12·6340, per cent.

The petroleum ether extract was a yellowish soft waxy mass consisting of sterols, fats, etc. The ethereal solution was a dark soft mass consisting chiefly of organic acids. The chloroform extract was a dark mass, giving a faint reaction for alkaloids but consisted chiefly of organic acids. The alcoholic extract was a dark mass whose aqueous extract showed a negative ferric chloride reaction but gave a precipitate with Mayer's reagent.

Extraction of the drugs and separation of potassium nitrate.

The dried and powdered drugs were extracted on a large scale by hot percolation with rectified spirit. The alcohol was recovered by distillation and the extractive weighed. A known weight of the dark green extractive was repeatedly extracted with hot distilled water and the aqueous extract filtered. The filtrate was concentrated under reduced pressure on a water-bath and on cooling a large mass of crystals, consisting chiefly of potassium nitrate, separated out. They were filtered off and the mother-liquor concentrated further under reduced pressure. On cooling, another crop of crystals was obtained. This was filtered off and combined with the first crop and weighed after drying in a desiccator.

1. *Bœrhaavia diffusa.*—About 10 kilo of the dried plant gave about 598 grammes of extractive, and 479 grammes of extractive (equivalent to 8 kilo of the drug) gave 42 grammes of crystals of salts, chiefly potassium nitrate, corresponding to about 0·52 per cent of the original dry plant.

2. *Trianthema portulacastrum* (white flowered).—About 9 kilo of the dried plant gave about 500 grammes of extractive which yielded 169 grammes of crystals of salts, chiefly KNO_3, corresponding to about 1·88 per cent of the original dry plant.

3. *Trianthema portulacastrum* (red flowered).—About 10 kilo of the dried plant gave about 1,250 grammes of extractive which yielded 235 grammes of crystals of salts, chiefly KNO_3, corresponding to about 2·35 per cent of the original dry plant.

Estimation of potassium nitrate in the salts isolated from the extractives and the direct estimation of KNO_3 in the original drug.

For the estimation of nitrates in the salts isolated from the extractives, 0·2 gramme of the crude salt was dissolved in 2 c.c. of water, filtered and washed from impurities and the filtrate evaporated to dryness. The residue was taken up with 1 c.c. of water, transferred to a Lunge nitrometer and the nitrate estimated as described below:—

For the direct determination of nitrate in the plant, 100 grammes of the dry powdered drug was thrice macerated with cold water, filtered through cotton-wool and the extract evaporated to dryness. The residue was taken up with rectified spirit, the extract evaporated to dryness and taken up with water and made up to 30 c.c. Three c.c. of the aqueous solution was evaporated to dryness, the residue dissolved in 1 c.c. of water and transferred to a Lunge nitrometer. The nitric oxide liberated by shaking the solution with sulphuric acid in the presence of mercury was measured and the amount of nitrate calculated as KNO_3 was found out from the volume of NO thus obtained.

The nitrates, calculated as KNO_3, from the crude salts isolated from the extractives were as follows:—

	Per cent.
B. diffusa	0·33
T. portulacastrum (white) ..	0·95
T. portulacastrum (red) ..	2·50

The nitrates, calculated as KNO_3, obtained by the direct determination from the whole plant were as follows:—

	Per cent.
B. diffusa	0·36
T. portulacastrum (white) ..	1·71
T. portulacastrum (red) ..	2·64

It may be mentioned here that the figures obtained from the crude salts are not strictly comparable, since the extraction and separation of the salts were not done in a quantitative manner. The results of the direct determination of the nitrates are, however, very interesting, showing the large difference between *T. portulacastrum* (red) and that of *B. diffusa*.

Isolation of the alkaloid.

The mother-liquor obtained after filtering off the two crops of crystals of crude KNO_3 was made slightly alkaline with ammonia in order to liberate the free bases and then extracted repeatedly with chloroform until the extract gave no tests for alkaloids. The total chloroform extract was carefully de-hydrated over potassium carbonate filtered and evaporated to dryness. The brownish pasty residue was dissolved in a small quantity of dry chloroform and precipitated with dry ether. The process was repeated. The residue was then dissolved in a small quantity of absolute alcohol and precipitated with dry ether and dried *in vacuo* over calcium chloride, when it was obtained as a cream-coloured powder melting with decomposition at about 175°C. after shrinking at a lower temperature. A portion was dissolved in absolute alcohol and allowed to crystallize slowly. Some colourless crystals separated out after a few days but they could not be isolated from the main mass of the amorphous base. The base was found to be soluble in water. The relative weights of the alkaloids thus isolated were as follows:—

1. *B. diffusa.*—Eight kilo of the drug gave 3·54 grammes of the total unpurified alkaloid, showing an yield of about 0·044 per cent.

2. *T. portulacastrum* (white).—Nine kilo of the drug gave 2·14 grammes of the total unpurified alkaloid, showing an yield of about 0·024 per cent.

3. *T. portulacastrum* (red).—Ten kilo of the drug gave 4·898 grammes of the total unpurified alkaloid, showing an yield of about 0·049 per cent.

A comparative study of the alkaloids found in the three drugs.

The crude alkaloids isolated from the three drugs resinified easily and could not be crystallized in any workable quantity for further chemical analysis. All of them had the same bitter taste and were soluble in water, alcohol and chloroform, but less so in ether. They gave precipitates with Mayer's reagent, picric acid, platinic chloride and other alkaloidal reagents. All the bases crystallized from alcohol in colourless plates. All the three picrates, which were prepared by precipitating an aqueous solution of the base with a saturated aqueous solution of picric acid, washing the picrate by decantation and finally over a porous plate and dried over calcium chloride in a desiccator and formed yellow amorphous powders, showed about the same melting point; thus, the picrates from *T. portulacastrum* (white) and *T. portulacastrum* (red) softened at about 102°C. and melted with decomposition from 118°C. to 120°C., while the picrate from *B. diffusa* softened at about 93°C. and melted with decomposition at 117°C. Again, the platinic chlorides, which were prepared by precipitating the aqueous solutions of the bases with a 10 per cent aqueous solution of platinic chloride, washed by decantation and dried over porous plate in a desiccator over calcium chloride, formed orange-yellow crystalline powders, having about the same melting points. Thus, the platinic chlorides from *T. portulacastrum* (white) and *T. portulacastrum* (red) softened at about 113°C. to 116°C. and melted with decomposition at 121°C. to 122°C. and that from *B. diffusa* softened at about 110°C. and melted with decomposition at 119°C. to 120°C. All these common characteristics show that the alkaloid present in all these three drugs is the same.

Isolation of the water-soluble bases.

The alkaline mother-liquor left after exhaustive extraction of the alkaloid with chloroform was warmed on the water-bath to remove the excess of ammonia. It was then treated with sulphuric acid so as to make a final concentration of 5 per cent of H_2SO_4. The water-soluble bases were precipitated with a 20 per cent solution of phosphotungstic acid in 5 per cent sulphuric acid. The precipitate was allowed to settle, filtered and washed with 5 per cent sulphuric acid till free from phosphotungstic acid. The precipitate was suspended in a mixture of 3 volumes of acetone and 4 volumes of water and treated with baryta water in order to liberate the bases. When the decomposition was complete the barium phosphotungstate was filtered off and washed thoroughly with hot water until the washings no longer gave any precipitate with the solution of phosphotungstic acid in sulphuric acid. Carbon dioxide was passed through the filtrate to remove the excess of barium, filtered and the solution evaporated to dryness in vacuum. The residue was extracted with absolute alcohol and filtered. The process was repeated until the residue from the alcoholic extract was free from barium. The bases were finally obtained as a brownish-yellow mass, easily soluble in water. They did not show any marked pharmacological action and they were not therefore investigated further.

The comparative yields of the water-soluble bases were as follows :—

B. diffusa.—Eight kilo of the drug gave 2·3050 grammes of the bases, showing an yield of about 0·0288 per cent.

T. portulacastrum (white).—Nine kilo of the drug gave 2·6782 grammes of the bases, showing an yield of about 0·0297 per cent.

T. portulacastrum (red).—Ten kilo of the drug gave 3·1698 grammes of the bases, showing an yield of about 0·0317 per cent.

Summary and conclusions.

The three plants, *Bœrhaavia diffusa* Linn. and the white and red flowered 'varieties' of *Trianthema portulacastrum* Linn., are used indiscriminately in different parts of India as the Ayurvedic drug Punarnava. The former belongs to the family Nyctaginaceæ and the latter to Ficoideæ. This common confusion is very unfortunate, but the present comparative chemical examination has revealed some redeeming features. Although they belong to two different families, the chemical constituents which may account for their medicinal properties are common. Both contain comparable quantities of potassium salts, mainly potassium nitrate, *T. portulacastrum* (red) showing the highest percentage of nitrates and *B. diffusa* the least. Both contain the same water-soluble active alkaloid, punarnavine, *T. portulacastrum* (red) and *B. diffusa* showing similar amounts, while *T. portulacastrum* (white) showing less. The total extractives were the highest in the case of *T. portulacastrum* (red) and least in the case of *B. diffusa*. The other water-soluble bases, which were comparatively inactive, showed similar amounts in all the three plants. The similarity in the chemical constituents in plants belonging to different families is, therefore, very striking and is a redeeming feature in their common use in spite of the confusion that has hitherto existed in the use of the drug called Punarnava.

In conclusion, we desire to express our thanks to Mr. J. K. Lahiri, M.Sc., for carrying out the different estimations of the nitrates and to Mr. A. T. Dutt, B.Sc., for preparing the derivatives of the alkaloids for their comparison.

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STUDIES ON PEPTIC ULCER IN SOUTH INDIA.

Part II.

A STATISTICAL SURVEY.

BY

MAJOR J. R. DOGRA, M.D., I.M.S.

(Work done under the Indian Research Fund Association.)

(From the King Institute, Guindy, Madras.)

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A STATISTICAL survey of peptic ulcer has not hitherto been made in this country, perhaps on account of the innumerable difficulties in the way. In such a country as India, with its widely scattered village population, consisting of illiterate and ignorant humanity, with lack of easy means of transport, it is not a matter of mere conjecture to state that a vast majority of such village folk never receive any medical relief, as the term is understood in civilized countries of the world. Bradfield (1938) estimated that there were 35,000 to 40,000 qualified doctors practising in India, i.e. 1 doctor for every 10,000 of the population. A large proportion of the medical profession is confined to urban areas, with the result that the villager has either to do without any medical aid or has to go to the nearest big town for relief. It must, therefore, be borne in mind that any data in which a village-to-village survey is not included, do not give a correct estimate of the prevalence and distribution of any particular disease, even when all centres of medical relief are included.

Until recently, peptic ulcer was not recognized clinically. Rogers (1914), in a study of 1,000 post-mortem examinations at Calcutta, found peptic ulcer in only 0·4 per cent of autopsies. The error in clinical diagnosis was 100 per cent. In spite of a very large number of cases of ‘ dyspepsia ’ treated all over the country, it is not recognized that this symptom complex may, in a vast majority of cases, be the result of ulceration of the stomach and duodenum, with the result that a large number of these cases are missed, and those that do apply for treatment are often seen at a very advanced stage of peptic ulceration.

In South India, although dyspepsia has been known from time immemorial, it was not until comparatively recently that its significance was realized. At the Government General Hospital, Madras, Molesworth (1895) treated as many as 100

in-patients in one year. He described his cases as follows: 'In the great majority of cases this condition was very chronic and associated with dilatation of the stomach varying from slighter forms to enormous distension of that organ. Most of the patients hailed from the West Coast. Daily lavation with warm water and permanganate of potash proved most beneficial'. No reference is made to any operative procedure adopted for these cases. Similar conditions prevailed in subsequent years until ten years later, when Niblock (1905) performed the first gastro-jejunostomy for pyloric obstruction. He found that 'the pylorus was contracted apparently as a result of ulcer near the pyloric orifice'.

In the extreme south of the peninsula, Pugh (1913) of the London Mission Hospital, Neyyoor, South Travancore, described six cases operated on by himself and his colleagues during 1912. The operations were done for 'cicatricial stenosis of the pylorus and duodenum'. Pugh met with a great deal of opposition from some of his colleagues to this surgical treatment for 'dyspepsia'. A similar attitude is maintained even to this day by some members of the medical profession in the areas surveyed.

In addition, there is the well-known reluctance of the people to submit to operative treatment for any condition whatever. This is quite understandable amongst people, ignorant and poor, and with a philosophy of life resulting from doctrines of theistic fatalism. The result is that a large number of cases continue to suffer from dyspeptic symptoms for long periods of time, either without applying for treatment, except that of indigenous systems of medicine, or delaying it until the symptoms become sufficiently aggravated to force the patients to go to the nearest centre for medical relief.

The medical centres where adequate facilities for diagnosis and treatment of this disease are available are few and far between, so that the patients have to travel great distances to reach them. Consequently, it must be borne in mind that the number of cases treated at a particular centre does not necessarily indicate the incidence of the disease in the area where the centre is located. A study of 258 cases at Madras showed that of these only 54 cases were from Madras proper. The remainder were from out-stations. During 1938, the London Mission Hospital, Neyyoor, treated 227 cases of this disease of which only one case was from Neyyoor itself.

Despite the difficulties described above a study of the disease in different areas in South India has been made, in order to ascertain the common features of the malady in such vast territories amongst people who not only show racial differences but present a great variety of culture, diet, habits, etc. so that research into the ætiological factor or factors may be directed along the right lines.

Material and methods.

Thanks to the pioneer work of Bradfield and Somervell in South India, since 1922, there have now developed several large centres, namely, at Vizagapatam, Madras, Madura and Neyyoor, where every year a large number of cases apply for treatment for dyspeptic symptoms. Modern methods of diagnosis and treatment are available at all these centres. Surgeons of renown have established the reputation of

these institutions and have thus gained the confidence of even the ignorant and the poor. All these centres were visited by the writer and a large number of case records examined. The present communication is a statistical account of this survey.

Furthermore, at each centre, a detailed study of cases operated on was made from the actual case sheets and not from any compilation of hospital statistics. In addition to conference with the local surgeons and physicians and examination of actual cases of this disease in the hospital at the time, a study of the people, their environment, economic state, diet, etc. was made. Data regarding different areas visited, including population figures, religious group distribution, etc. have been taken from the Census Report published after the census of 1931 and from the Imperial Gazetteer.

The entire region covered by the province of Madras and the states of Mysore, Travancore and Cochin has been included under the term South India. The four main population groups in this region, namely, the Malayalees in Travancore and Cochin, Canarese in Mysore, Tamilians in Madura and Madras, and the Andhras in the Northern Circars are dealt with separately.

TRAVANCORE.

Forming an irregular triangle with its apex at Cape Comorin, the southernmost Indian State of Travancore is bounded on the north by the state of Cochin and the British district of Coimbatore, on the east by the Western Ghâts and the districts of Madura, Ramnad and Tinnevelly, while on the south and west it is washed by the Indian Ocean and the Arabian Sea. The mountains which separate it on the north and east from British India are clothed with magnificent primeval forest, while the remainder of the country is covered with dense plantations of coco-nut palms which, in a great measure, constitute the wealth of the country. The whole surface is undulating and presents a series of hills and valleys traversed from east to west by numerous rivers. Most of these rivers are navigable for some distance inland and, after a tortuous course, flow into the backwaters of the sea.

The hilly region is very extensive. The mountains possess every variety of climate and vegetation and have numerous tea and rubber plantations. The elevation varies, the loftiest—the Anamudi Peak (8,837 feet)—being the highest in India south of the Himalayas. The southern end of the state has extensive rice cultivation with clusters of houses and palmyra groves resembling, in some respects, the neighbouring district of Tinnevelly.

An interesting chain of lakes or backwaters extends along the coast from the northernmost frontier to about 20 miles south of Trivandrum. These are either the expansions of the rivers at their mouths, or extensive sheets of water receiving the accumulated flow of several streams and rivers. A strip of land from seven miles to about half a mile wide separates these backwaters from the sea. There are, however, several outlets from them to the sea and they are all inter-connected and form a very important means of communication. In addition, the bulk of the country has been opened up by a network of roads and canals. In Central and South Travancore, there is a mile of road to almost every square mile of the country.

One railway line about a hundred miles in length now cuts across the state from east to west and thence runs along the coast from north to south.

The climatic variations of the whole of India may be experienced, though a warm humidity is one of the special features of the climate. At the higher elevations, the climate is always cool and even cold at certain seasons. From March to May the heat is oppressive in the low lands, although the temperature seldom rises above 90 degrees in the shade.

Of the total area of 7,625 square miles, 2,500 are covered with forest, jungle and backwaters, and about 2,000 (a portion of which is available for pasturage) by small hills. According to the 1931 census, about five million people inhabit this 4,500 square miles of land and consequently Travancore is one of the most densely populated of countries. In India Cochin alone, and outside India, only Java, Malaya, England, Wales and Belgium have slightly higher densities than Travancore. Most of the population is restricted to the strip of land between the Ghâts and coast line.

According to the 1931 census Hinduism is the predominant religion of the state, constituting the belief of about two-thirds of the entire population. Christianity comes next with over a fourth of the entire population. The Mohammedan religion claims a sixth of the population and ranks third.

Twenty-nine languages were returned at the 1931 census, of which Malayalam is the mother-tongue of 84 per cent and Tamil of 15 per cent of the entire population, the other 27 languages being spoken by the remaining 1 per cent. Excluding children under five years, the number of literates per thousand of population is 289. For males and females separately the figures are 408 and 168 per thousand respectively. In education, therefore, Travancore occupies the foremost position when compared with British India and other Indian States.

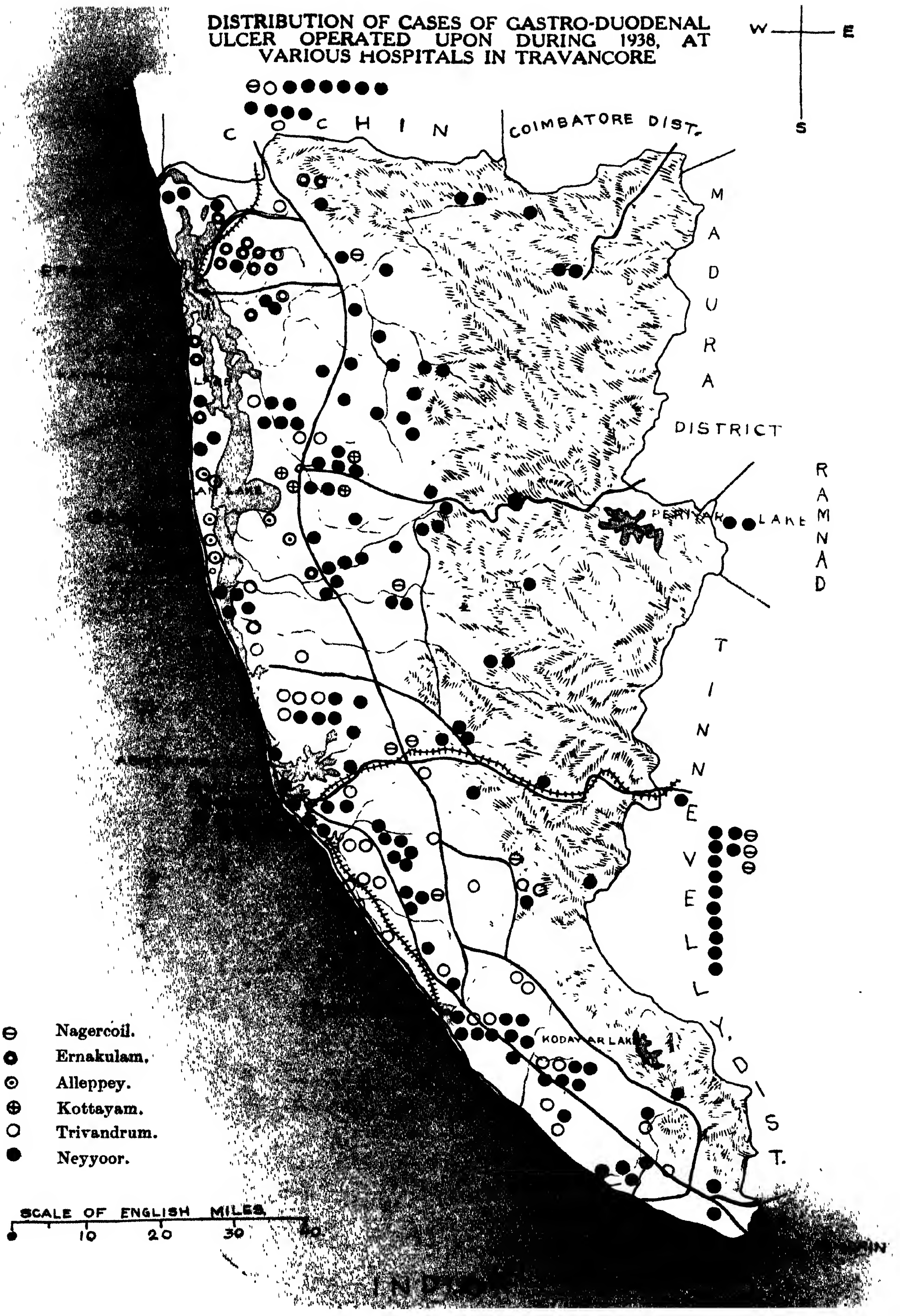
Travancore is essentially an agricultural country with this difference, that here the proprietors and the tenants of the land mostly live on the land itself. The chief food grain of the country is rice (*Oryza sativa*), although great millet or cholam (*Sorghum vulgare*) and raghi (*Eleusine coracana*) are also grown. As in other parts of South India, rice is the staple article of food, but in Travancore, tapioca root supplements it to a considerable extent. A large proportion of the people live in small mud-huts. With small holdings, their income is small and poverty is the rule.

Travancore is well supplied with state and state-aided hospitals. Most of the state and mission hospitals were visited. It may be mentioned here that the easy means of transport, the greater degree of literacy and the facility with which surgical treatment for peptic ulcer is available, have greatly reduced the number of cases which do not apply for operative treatment, so that the total number of cases operated on almost represents the actual incidence of the disease. In this respect Travancore differs from other parts of South India surveyed in this paper.

Distribution of the cases of peptic ulcer in Travancore.

The distribution of proved cases of gastro-duodenal ulcer operated upon at Nagercoil, Neyyoor, Trivandrum, Kottayam, Alleppey and Ernakulam during the year 1938 is shown in Map 1.

DISTRIBUTION OF CASES OF GASTRO-DUODENAL ULCER OPERATED UPON DURING 1938, AT VARIOUS HOSPITALS IN TRAVANCORE



A uniform distribution of the peptic ulcer cases is evident. Their prevalence corresponds to the distribution of population. There is no evidence to support Somervell and Orr's view (1936), that the disease shows regional distribution.

Statistical analysis.

At each centre three years' records were studied for the analysis given below :—

(*a*) The relative frequency with regard to the site of ulcer in cases from the two main centres, i.e. the State General Hospital, Trivandrum, and the London Mission Hospital, Neyyoor, is shown in Table I. It may be mentioned here that at Neyyoor cases apply for treatment from all over the state and the neighbouring districts of the province of Madras (*see* Map 1). This is obviously due to the extreme popularity and reputation of this hospital for gastric surgery.

TABLE I.

Site of ulcer in 787 *proved cases of gastro-duodenal ulcer operated upon during* 1936, 1937 *and* 1938, *at the London Mission Hospital, Neyyoor, and the State General Hospital, Trivandrum.*

Site of ulcer.	1936.	1937.	1938.	Total.
Duodenum	178	240	240	658
Duodenum and pyloric antrum ..	7	26	34	67
Duodenum and stomach (lesser curvature).	2	3	3	8
Pyloric antrum	9	12	15	36
Stomach (lesser curvature) ..	3	11	4	18
TOTAL ..	199	292	296	787

It would appear from the above table that ulcer of the lesser curvature of the stomach occurred very rarely and that duodenal ulcer was 36 times more common.

(*b*) *Age and sex incidence.*—The figures include all the hospitals visited during this investigation in Travancore (*see* Table II).

TABLE II.

Age and sex incidence in 964 cases of gastro-duodenal ulcer collected from Travancore.

Age groups.	Males.	Females.	Total.
0– 9 ..	..	..	..
10–15 ..	4	3	7
16–20 ..	33	4	37
21–25 ..	94	6	100
26–30 ..	158	6	164
31–35 ..	153	6	159
36–40 ..	158	13	171
41–45 ..	127	3	130
46–50 ..	107	2	109
51–55 ..	40	3	43
56–60 ..	31	1	32
61–65 ..	8	..	8
66–70 ..	4	..	4
TOTAL ..	917	47	964

(*c*) *Religious groups.*—Taking Travancore State as a whole, the total number of proved cases, for three years, of gastro-duodenal ulcer collected from all the hospitals visited, when compared with the population figures as returned in the

Census Report of 1931, showed rough agreement as regards numerical distribution among the various religious groups, as shown in Table III :—

TABLE III.

Religious group distribution in 886 cases of gastro-duodenal ulcer collected from Travancore.

Religious groups.	Census 1931.	Per cent.	Ulcer cases.	Per cent.
Hindus	3,134,888	61·11	604	68·28
Christians ..	1,604,475	31·90	218	24·60
Mohammedans ..	353,274	6·95	63	7·11
Others	3,336	0·04	1	0·01
TOTAL ..	5,095,975	100·00	886	100·00

(*d*) *Occupation and economic state.*—The majority of cases were from amongst the lowest strata of society and therefore extremely poor. Of the 116 cases investigated from this aspect, 55 per cent were agriculturists or agricultural labourers and the remainder petty merchants, weavers, beggars, destitutes, etc. The disease was found to be extremely rare amongst the well-to-do.

MYSORE STATE.

The State of Mysore, consisting of an undulating table-land much broken up by chains of rocky hills and scoured by deep ravines, forms a triangle with its apex to the south, at the point where the Western Ghâts and the Eastern Ghâts ranges converge. It is bounded by districts of the province of Madras on all sides except on the north-west where it is bordered by Bombay province and towards the south-west where Coorg intervenes. The general elevation rises from about 2,000 feet above sea-level along the northern and southern frontiers to about 3,000 feet at the central water-shed which separates the basin of the Kistna in the north from that of the Cauvery in the south.

Broadly speaking, Mysore is divided into two regions of distinct character: the hilly country in the west and the more open country in the east comprising the greater part of the state. Here, the wide-spreading valleys and plains are occupied by numerous villages and populous towns.

Climatically, Mysore has three seasons: the rainy, the cold and the hot. The first commences with the bursting of the south-west monsoon, generally early in June, and continues, with some intervals in August and September, to the middle of November, closing with the heavy rains of what is called the north-east monsoon. It is followed by the cold season with its 'endless sunshine with a tinge of chilliness which is almost bracing'. The cold weather lasts till the end of February. The hot weather sets in with March and is at its worst by the end of May. The annual rainfall varies. The excessive rains of the hills diminish eastwards, and from 20 to 37 inches may be accepted as the general average for the greater part of the state.

The population of the state is about six millions, of which the Hindus constitute 91·74 per cent. Of the rest, 6·08 per cent are Mohammedans, 1·33 per cent Christians and 0·85 per cent belong to other religions. Nearly three-fourths of the population is employed in agriculture.

The staple food grains are raghi (*Eleusine coracana*), cholam (*Sorghum vulgare*), rice (*Oryza sativa*), and millets (*Panicum miliaceum*), gram (*Phaseolus mungo*) and other pulses. Oil seeds include gingelly (*Sesamum indicum*) and castor (*Ricinus communis*), among spices may be mentioned capsicum, ginger, coriander, cumin seed, etc. Tobacco, mustard, onions, garlic, etc. are also cultivated. Fruit and vegetable production has received special attention in the neighbourhood of Bangalore from where these products are exported to the whole of South India.

The total area of 29,326 square miles covered by the State of Mysore has a population density of 224 per square miles, but it must be remembered that Mysore district has the largest population. Bangalore is the second in point of population. Chitaldrug, Tumkur, Shimoga and Kolar districts cover larger areas but have a smaller population. Kadur is the least populated.

Mysore is fairly well supplied with hospitals but the main centres where operative work is done are Krishnarajendra Hospital, Mysore, Victoria Hospital, Bangalore, and the Government Hospital, Kolar Gold Fields. The two former hospitals were visited. It may be noted that the means of transport are cheap but inadequate, the people are not as well educated as in Travancore and, except for the three medical centres mentioned above, facilities for much operative work are meagre. At the mofussil stations diagnosis of peptic ulcer is likely to be difficult.

Distribution of the cases of peptic ulcer in Mysore.

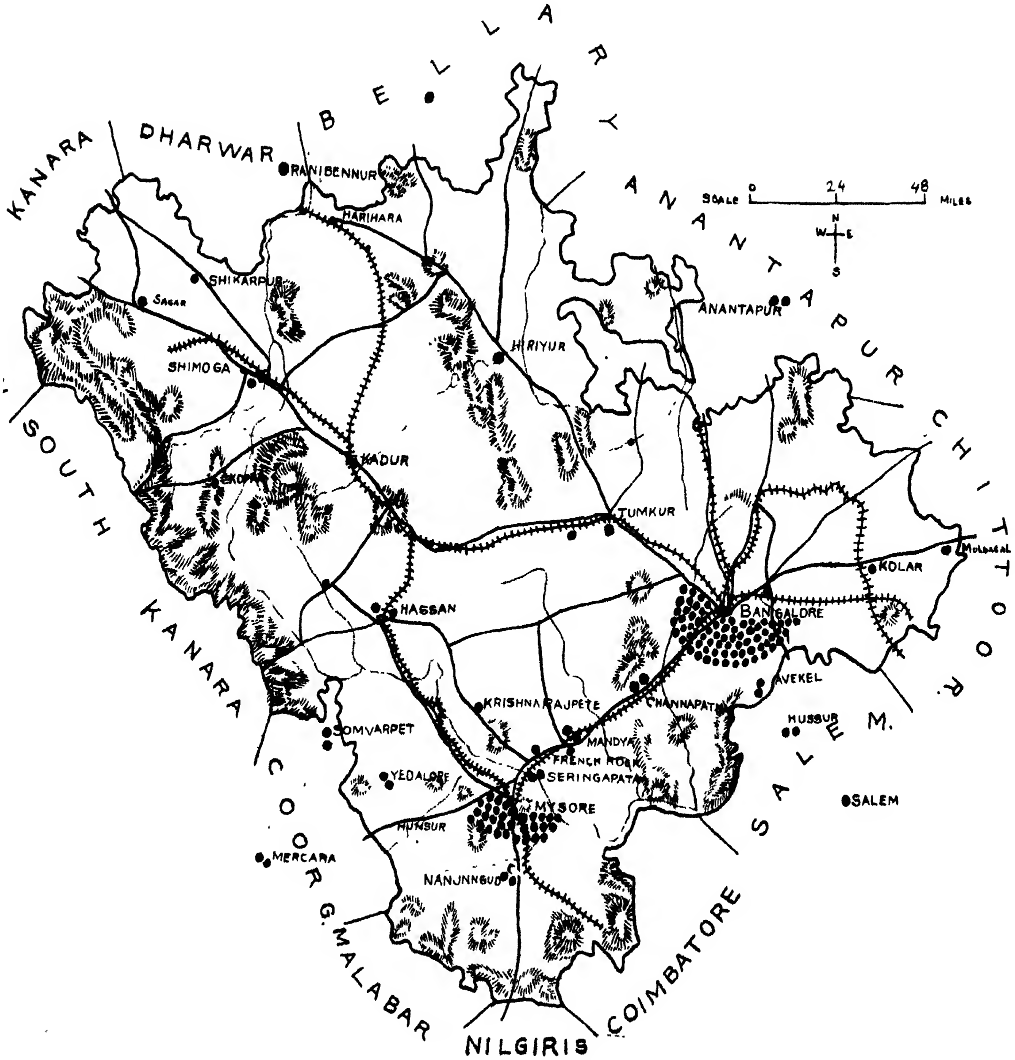
The distribution of peptic ulcer cases diagnosed and treated during the year 1939 at the Krishnarajendra Hospital, Mysore, and Victoria Hospital, Bangalore, is shown in Map 2. It may be noted that these cases were not all proved cases, inasmuch as all of them were not operated upon but, owing to adequate facilities for diagnosis and the able staff of these institutions, one may presume the correctness of diagnosis in a very large percentage of cases.

It will be seen that the largest number of cases are distributed in the areas where the population is greatest, i.e. Bangalore and Mysore. Cases in the districts

MAP 2.

MYSORE STATE

DISTRIBUTION OF CASES OF GASTRO-DUODENAL ULCER (1939 CASES)



including the neighbouring districts of the province of Madras, are chiefly confined to the towns and villages connected to Mysore and Bangalore by rail and road, due to the fact that it is easy for the patients from these areas to reach the main medical centres. However, no region seems entirely exempt from this disease.

Statistical analysis.

Three years' records (1937 to 1939) at the two hospitals mentioned above were studied for the analysis given below. Of the total surgical admissions, the percentage of gastro-duodenal ulcer cases was 2·95. A study of the actual case records, however, revealed that a large proportion of these cases refused operation. Of the 533, only 144 agreed to operative treatment.

It is regretted that detailed description of the findings at operation was not available in some cases. The site of ulcer was not noted and therefore classification of these 144 cases according to site of ulcers is based on entries in the diagnosis column. Experience in Mysore, as at other centres, showed that the entries in the diagnosis column were not always reliable. This is perhaps due to the fact that this column is filled in before the operation, the nurse or student entering the word 'gastric'. In absence of any alteration in this column and detailed notes of the operative findings, the site of the ulcer remains problematical. For the purpose of the present classification these cases had necessarily to be considered as those of gastric ulcer. This, perhaps, is the reason why, relatively, the incidence of gastric ulcers is high in Mysore. Table IV shows the cases operated on, classified according to the site of the ulcer :—

TABLE IV.

Site of ulcer in 144 proved cases of gastro-duodenal ulcer operated upon during 1937, 1938 and 1939 at the Krishnarajendra Hospital, Mysore, and Victoria Hospital, Bangalore.

Site of ulcer.	Krishnarajendra Hospital.	Victoria Hospital.	Total.
Duodenum	33	50	83
Duodenum and pyloric antrum ..	2	10	12
Duodenum and stomach (lesser curvature).	1	..	1
Pyloric antrum	7	19	26
Stomach (lesser curvature) ..	7	15	22
TOTAL ..	50	94	144

From Table IV it would appear that ulcer of the lesser curvature of the stomach occurs more frequently in Mysore. This, however, is not believed to be the true state of affairs by the author for the reasons given above.

The age and sex incidence and religious group distribution of gastro-duodenal ulcer cases is shown in Tables V and VI respectively :—

TABLE V.

Age and sex incidence in 503 cases of gastro-duodenal ulcer collected from Mysore.

Age groups.	Males.	Females.	Total.
0– 9 ..	..	..	..
10–15 ..	1	..	1
16–20 ..	28	2	30
21–25 ..	62	3	65
26–30 ..	99	9	108
31–35 ..	68	6	74
36–40 ..	76	8	84
41–45 ..	48	5	53
46–50 ..	50	5	55
51–55 ..	10	1	11
56–60 ..	17	2	19
61–65 ..	3	..	3
TOTAL ..	462	41	503

TABLE VI.

Religious group distribution in 503 cases of gastro-duodenal ulcer collected from Mysore.

Religious groups.	Census 1931.	Per cent.	Ulcer cases.	Per cent.
Hindus	6,016,000	91·74	387	76·94
Mohammedans ..	399,000	6·08	102	20·28
Christians ..	88,000	1·33	14	2·78
Others	56,000	0·85	..	0·00
TOTAL ..	6,557,000	100·00	503	100·00

Occupation and economic state.—Of the 231 instances in which occupation was known, 81, i.e. about 35 per cent, belonged to the labouring classes, including agriculturists, agricultural labourers and mill workers. The remainder were petty merchants, servants and workers from urban areas of Bangalore and Mysore. The smaller number of agriculturists in this group is presumably due to the fact that these folk do not come for treatment from distant areas. No cases were admitted to the paying patients' wards of the state hospitals for this malady during the three years under review and it may thus be stated that the disease is confined chiefly to the poorer strata of society.

MADURA DISTRICT.

The district of Madura belongs to the province of Madras and is situated in its southern portion. It is bounded on the north by the districts of Trichinopoly and Coimbatore, on the south and south-east by Ramnad district ; on the west by the State of Travancore. Part of its south-western and western border abuts on the Western Ghâts. Its general aspect is that of a level plain sloping gradually eastwards. The chief hills on the west are the Palni hills, parallel to these are the

Varushanad hills and the Andipatti ranges from the Western Ghâts running in a north-easterly direction. Between them is the Cumbum valley, well wooded and green, kept green by perennial streams.

The climate of the district is hot, dry and variable. There is no real cold season but it is less warm from November to February. It is, however, cooler in Dindigul and the Cumbum valley. The climate on the upper Palni hills, according to the Gazetteer, 'is probably one of the finest in India'. The annual rainfall of the district as a whole, excluding the Palnis, varies from 26 to 36 inches, averaging about 30 inches. Of this more than half is registered during the north-east monsoon in the last three months of the year, about one-fourth during the four months of the south-west monsoon from June to September and about one-seventh during the months of April and May.

The total area of 4,912 square miles is inhabited by 2,193,747 inhabitants. The distribution of the population is not uniform. The majority of the population depends on agriculture for occupation and livelihood. Large urban centres are situated along the main rail and road routes. Madura is the principal town, the second largest town in the province of Madras with a population of 186,000. Madura is a large industrial centre with a silk fabric industry and cotton mills with many mill workers. The inhabitants of the district are chiefly Tamilians.

The staple food grains cultivated are rice (*Oryza sativa*), varughu (*Paspalum scrobiculatum*), cholam (*Sorghum vulgare*), raghi (*Eleusine coracana*), and cambu (*Pennisetum typhoideum*). On the hills there are some tea and coffee plantations. Generally speaking, the agricultural labourer shares the fate of his class in other parts of South India in being ignorant and poor.

At Madura, the Government Headquarters Hospital and the two American Mission Hospitals are the main modern centres for medical relief for the inhabitants of the entire district. These were visited and the statistics available studied.

The distribution of the case of peptic ulcer in Madura district.

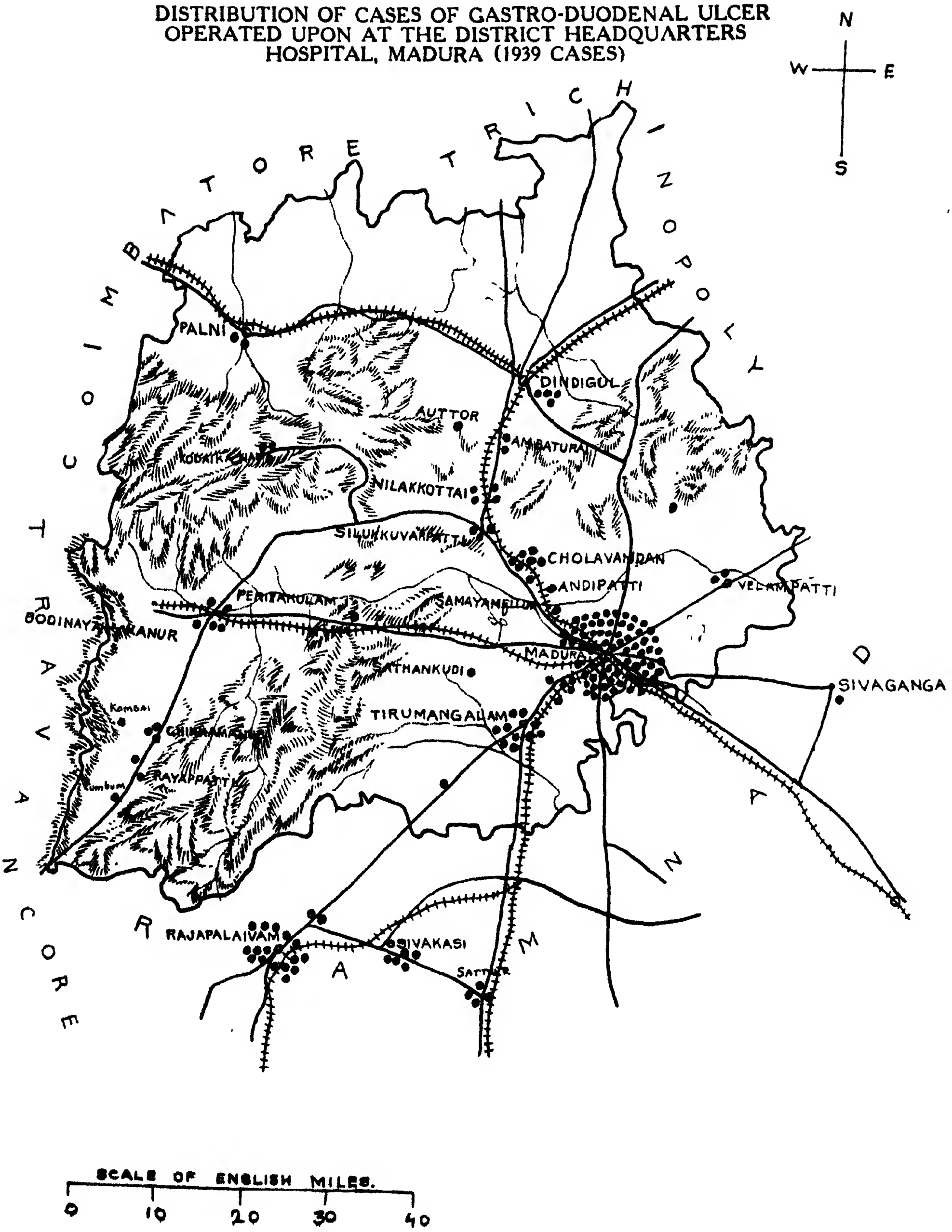
The distribution of gastro-duodenal ulcer cases admitted to the District Headquarters Hospital, Madura, and the Willis F. Pierce Memorial Hospital, Madura, is shown in Map 3. Of the 390 cases, 99 were homeless and gave no address, 43 were from the neighbouring district of Ramnad, 11 from Tinnevelly, 8 from Trichinopoly, 2 from Salem, 4 from Coimbatore and 4 from Tanjore.

The majority of the cases were from Madura itself. The remainder were from villages and towns situated on the main rail and road routes. It may be noted that Madura is nearer to some important places in the Ramnad district than the Headquarters Hospital at Ramnad itself, consequently a large number of cases from this district go to Madura (*see* Map 3).

MAP 3.

MADURA DISTRICT

DISTRIBUTION OF CASES OF GASTRO-DUODENAL ULCER OPERATED UPON AT THE DISTRICT HEADQUARTERS HOSPITAL, MADURA (1939 CASES)



Statistical analysis.

From very carefully maintained records, a total number of 281 proved cases were studied. Classification of these cases according to the site of ulcers is shown in Table VII :—

TABLE VII.

Site of ulcer in 281 proved cases of gastro-duodenal ulcer operated upon during 1939 at the Headquarters Hospital, Madura, and W. F. Pierce Memorial Hospital, Madura.

Site of ulcer.	Headquarters Hospital.	W. F. P. M. Hospital.	Total.
Duodenum	245	2	247
Duodenum and pyloric antrum	5	..	5
Duodenum and stomach (lesser curvature).	2	..	2
Pyloric antrum	19	..	19
Stomach (lesser curvature) ..	8	..	8
TOTAL ..	279	2	281

It would appear that ulcer of the lesser curvature of the stomach occurs very rarely and that duodenal ulcer is 30 times more common.

The age and sex incidence and religious group distribution are shown in Tables VIII and IX :—

TABLE VIII.

Age and sex incidence in 380 cases of gastro-duodenal ulcer collected from Madura.

Age groups.	Males.	Females.	Total.
0– 9 ..	..	..	..
10–15 ..	7	..	7
16–20 ..	34	2	36
21–25 ..	53	2	55
26–30 ..	56	2	58
31–35 ..	50	6	56
36–40 ..	54	9	63
41–45 ..	35	4	39
46–50 ..	30	3	33
51–55 ..	14	2	16
56–60 ..	9	1	10
61–65 ..	5	..	5
66–70 ..	2	..	2
TOTAL ..	349	31	380

TABLE IX.

Religious group distribution in 380 cases of gastro-duodenal ulcer collected from Madura.

Religious groups.	Census 1931.	Per cent.	Ulcer cases.	Per cent.
Hindus	2,021,336	92·10	346	91·06
Mohammedans ..	90,587	4·10	19	5·00
Christians ..	83,769	3·80	15	3·94
Others	55	0·00	..	0·00
TOTAL ..	2,195,747	100·00	380	100·00

Occupation and economic state.—Of the 305 instances in which occupation was known, 243, i.e. 79 per cent, were agriculturists, agricultural labourers and mill workers. The well-to-do were extremely rare amongst those afflicted.

NORTHERN CIRCARS.

Under the term Northern Circars is included the area covering the districts of Vizagapatam, East and West Godavari, Kistna and Guntur. All these districts have several common features. (*a*) They occupy a narrow strip of alluvial land along the eastern coast of the Indian peninsula separated from the Nizam's Dominions and the Central Provinces by a range of hills—the main ranges of the Eastern Ghâts. (*b*) The two main rivers Godavari and Kistna spread out into their respective deltas accompanied by a large canal irrigation system resulting in abundance of water supply and consequent fertility of the soil. (*c*) The inhabitants of these districts are mainly Andhras who are distinctly different in culture, habits, diet, etc. from the inhabitants of the regions described already in this communication, i.e. the Tamilians, Canarese and Malayalees.

The districts of Godavari and Kistna with their rich soil chiefly cultivate rice which is pre-eminently the food grain of these districts. Next comes cholam (*Sorghum vulgare*), the pulses and maize, oil seeds, among which gingelly oil takes the first place, are also grown. Tobacco is raised throughout these two districts, while sugarcane is an important crop in the Guntur district.

In Vizagapatam district, the main crops are rice, cambu (*Pennisetum typhoideum*), raghi and cholam. The principal food grain of the masses is the 'dry crop', cambu and raghi. Generally speaking, inhabitants of this district use very highly spiced food with large quantities of chillies.

The hilly tracts of the districts are not thickly populated. Most of the population is distributed along the sea coast through which the main road and rail routes pass. The majority of the people are agriculturists, or agricultural labourers who work on the land.

There are several hospitals supported by Government and Mission agencies in each district. A comprehensive survey of this area was not made and only one centre, namely, King George's Hospital, Vizagapatam, was visited. Thus, all the cases treated at Cocanada, Rajahmundry, Ellore, Guntur and Masulipatam are not included. A study of cases from King George's Hospital, Vizagapatam, is recorded here.

The King George's Hospital, Vizagapatam, is the principal surgical centre in the Northern Circars. With its keen workers and adequate equipment, the hospital attracts a large number of cases from the neighbouring parts. Cases of gastro-duodenal ulcer admitted for treatment to this hospital during the year 1937 are shown in Map 4.

Statistical analysis.

Six years' records were studied, totalling 531 proved cases of gastro-duodenal ulcer. The classification of these cases according to the site of ulcer is shown in Table X. Age and sex incidence is shown in Table XI :—

TABLE X.

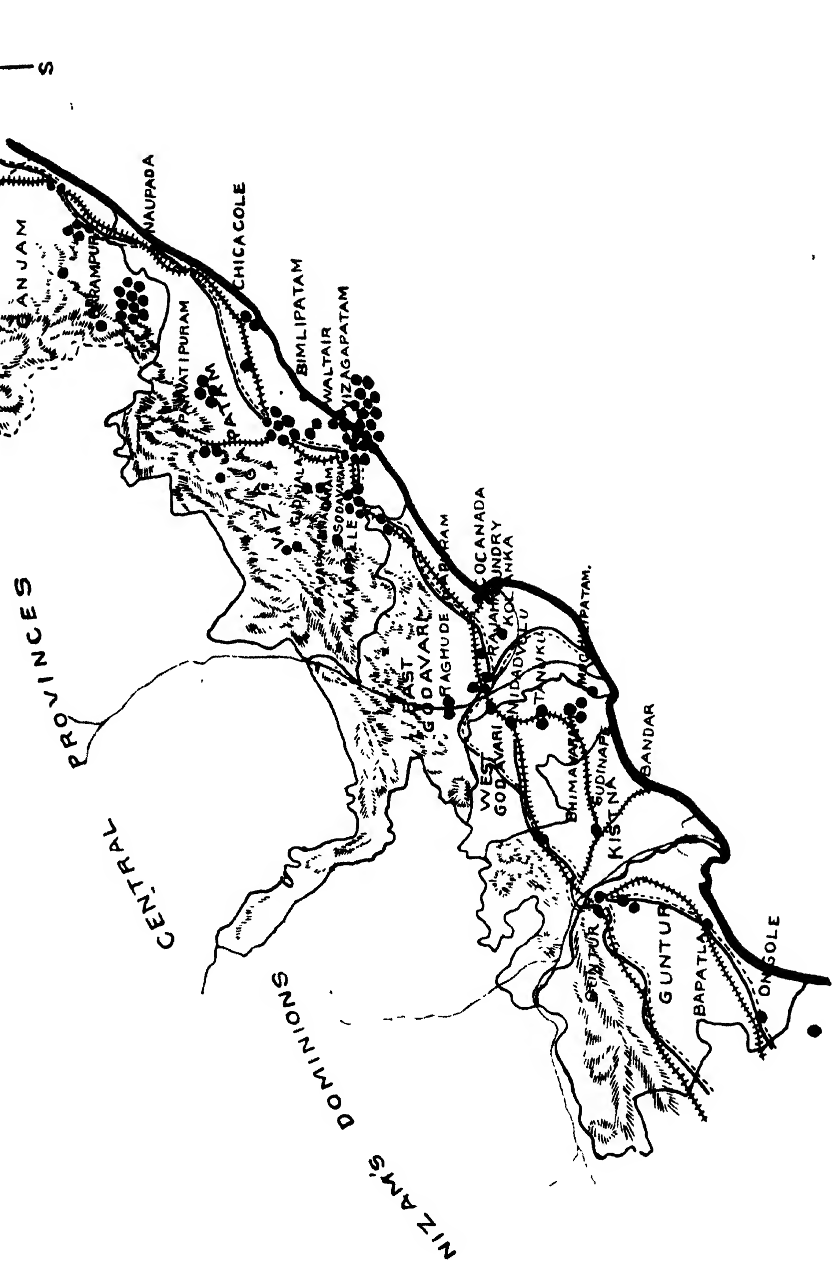
Site of ulcer in 531 proved cases of gastro-duodenal ulcer operated upon during 1932–37 at the King George's Hospital, Vizagapatam.

Site of ulcer.	1932.	1933.	1934.	1935.	1936.	1937.	Total.
Duodenum	63	78	64	56	54	48	363
Duodenum and pyloric antrum ..	10	13	24	20	15	11	93
Duodenum and stomach (lesser curvature).	1	7	1	2	3	3	17
Pyloric antrum	11	15	6	2	5	1	40
Stomach (lesser curvature) ..	1	5	5	2	2	3	18
TOTAL ..	86	118	100	82	79	66	531

MAP 4.

NORTHERN CIRCARS

DISTRIBUTION OF GASTRO-DUODENAL ULCER CASES OPERATED UPON AT K. G. HOSPITAL, VIZAGAPATAM (1937 CASES)



The proportion of duodenal to gastric ulcer in this series is 20 to 1. In a very carefully investigated series of 209 cases Kini (1940), from the same hospital, reports the proportion as 30 to 1.

TABLE XI.

Age and sex incidence in 524 cases of gastro-duodenal ulcer collected from the King George's Hospital, Vizagapatam.

Age groups.	Males.	Females.	Total.
0– 9 ..	..	..	..
10–15 ..	5	..	5
16–20 ..	38	4	42
21–25 ..	51	3	54
26–30 ..	124	7	131
31–35 ..	105	4	109
36–40 ..	97	10	107
41–45 ..	24	3	27
46–50 ..	32	1	33
51–55 ..	7	1	8
56–60 ..	6	..	6
61–65 ..	1	..	1
66–70 ..	1	..	1
TOTAL ..	491	33	524

Madras city.

The premier city with a population of 647,230 inhabitants has a large number of hospitals, the largest being the Government General Hospital, Madras. This hospital, being attached to the Medical College, is a great medical and surgical centre. The pioneer work of Niblock, Bradfield and Pandalai has established the reputation of this institution as the main centre for gastric surgery in South India. A very large number of operations on the stomach (three to four hundred) are performed every year. At this hospital, a study of the clinical features of 265 cases of gastro-duodenal ulcer was made and reported (Dogra, 1940). The number of cases in that series was considered too small for purposes of statistical evaluation and therefore ten years' records from the Senior Surgeon's clinic were examined in detail. It has already been pointed out that the cases treated at the Government General Hospital, Madras, came from all over the province but that the majority of them were from the neighbouring districts. In the present series of 1,895 cases, addresses were available in 540 only. The distribution of these, by districts, is shown in Table XII :—

Table XII.

Distribution of cases by districts in 540 proved cases of gastro-duodenal ulcer operated upon during the years 1929–38 by the Senior Surgeon at the Government General Hospital, Madras.

Districts.	Number of cases.	Districts.	Number of cases.
Vizagapatam	1	Chingleput	47
Godavari	1	South Arcot	39
Kistna	2	Salem	9
Guntur	6	Coimbatore	5
Nellore	10	Trichinopoly	7
Bellary	2	Tanjore	17
Anantapur	2	Madura	3
Cuddapah	6	Ramnad	1
North Arcot	40	Malabar	33
Chittoor	19	Madras	192
Tinnevelly	8	The Nilgiris	1
South Kanara	1	Travancore State ..	69
Mysore State	2	Cochin State	13
Pudukkottai State	1	Total ..	540

Table XII emphasizes the fact that the cases treated at the Government General Hospital, Madras, are mostly those from out-stations, and that the total number of cases is no indication of the incidence of the disease in Madras. Somervell (1940) and Rao (1938) use such hospital statistics for determining relative incidence of the disease. Their observations must necessarily be considered misleading.

Of the 19,814 surgical case records examined, i.e. ten years' (1929–38) records from the Senior Surgeon's clinic, 1,895 or 9·6 per cent were cases of gastro-duodenal ulcer. Of these 527 or 27·81 per cent either absconded or refused operation, 140 were cases of acute perforations and post-operative recurrence of symptoms. The remaining 1,228 proved cases of gastro-duodenal ulcer have been analysed with reference to (*a*) site of ulcer, (*b*) age and sex incidence.

(*a*) *Site of ulcer.*—Classification is shown in Table XIII :—

TABLE XIII.

Site of ulcer in 1,228 proved cases of gastro-duodenal ulcer operated upon during 1929–38 by the Senior Surgeon at the Government General Hospital, Madras.

Year.	Duodenum.	Duodenum and pyloric antrum.	Duodenum and stomach (lesser curvature).	Pyloric antrum.	Stomach (lesser curvature).	Total.
1929 ..	89	2	4	1	5	101
1930 ..	99	1	6	1	4	111
1931 ..	141	..	2	..	4	147
1932 ..	141	1	8	2	3	155
1933 ..	180	6	8	1	5	200
1934 ..	119	8	4	..	5	136
1935 ..	95	6	1	..	2	104
1936 ..	75	8	1	1	3	88
1937 ..	69	7	..	3	2	81
1938 ..	87	11	2	3	2	105
TOTAL ..	1,095	50	36	12	35	1,228

From Table XIII, the proportion of duodenal to gastric ulcer is 31 to 1.

(*b*) *The age and sex incidence.—See* Table XIV.

TABLE XIV.

Age and sex incidence in 1,099 *cases of gastro-duodenal ulcer collected from the Government General Hospital, Madras.*

Age groups.	Males.	Females.	Total.
0– 9 ..	..	..	..
10–15 ..	7	3	10
16–20 ..	60	..	60
21–25 ..	165	5	170
26–30 ..	246	5	251
31–35 ..	191	6	197
36–40 ..	169	8	177
41–45 ..	108	4	112
46–50 ..	69	1	70
51–55 ..	29	2	31
56–60 ..	16	..	16
61–65 ..	5	..	5
TOTAL ..	1,065	34	1,099

Occupation and economic state.—Of the 567 instances in which occupation was known, 335, i.e. 59·08 per cent, were agriculturists and agricultural labourers. The rest were weavers, blacksmiths, petty merchants, destitutes, etc.

Discussion.

In this statistical survey of peptic ulcer cases in Travancore, Mysore, Madura, Madras and the Northern Circars, several aspects of the disease have been elucidated which may now be briefly discussed under separate headings.

1. *The distribution of the disease.*—From the evidence available, it is justifiable to conclude that the disease is prevalent all over South India. In areas where adequate medical facilities are available, where there is a high degree of literacy and where easy and cheap means of transport are available, peptic ulcer cases occur uniformly in proportion to the population distribution, as in Travancore. In other regions, where surgical and medical centres are few and far between, the distribution of these cases is along the rail and road routes, and near large towns with their medical and surgical centres. There is no reason to believe, however, that peptic ulcer does not occur in villages far removed from large urban centres because no cases come from them. As was pointed out in the introduction to this paper, such village folk, owing to their ignorance and poverty, have neither the inclination nor the facilities for any modern methods of medical relief. The number of cases treated at a particular centre depends upon its situation, facilities for work, its reputation, the keenness of the medical staff and their renown as gastric surgeons. For these reasons, the number of cases of peptic ulcer at a given hospital is no guide to the incidence of this disease in the locality in which the hospital is situated. At the Government General Hospital, Madras, 'gastric cases' constitute about 10 per cent of total surgical admissions. When the geographical distribution of these cases is considered, it becomes evident that a large majority of these come from out-stations. The same is true in the case of the London Mission Hospital, Neyyoor.

2. *Site of ulcer.*—At each centre a study of a large number of case records has shown that duodenal ulcer—a slow-growing chronic ulcer of the first part of the duodenum—is the lesion universally encountered. Of the 2,971 proved cases 392 were duodenal ulcer occurring with pyloric or gastric ulcer, 2,478 were cases of duodenal ulcer and only 101 were cases of gastric ulcer of the lesser curvature of the stomach, i.e. duodenal ulcer was 25 times more common than gastric ulcer. It may, however, be stated that if the Mysore figures are excluded because of their doubtful accuracy then, of the 2,827 cases, there were 2,395 duodenal, 354 combined and 78 gastric ulcer cases, i.e. a proportion of 30 to 1. This is considered to be the correct proportion in which these cases occur in South India.

3. *The age incidence and duration of illness.*—To an average hospital class of patient, as to an average villager, the matter of time is of very little consideration. Births and deaths are similarly of little concern, particularly in relationship to dates. His daily time is relative to sunrise and sunset, whereas he measures the months in relation to crops which he cultivates. Amongst the more enlightened villagers the local calendar based on the Hindu or Mohammedan or any other era that may be in use, only confuses him when correlating it with the Christian era. His statements, therefore, with regard to his age and duration of illness cannot be relied upon. Much importance has thus not been attached either to age or the duration

of illness in these cases. From the data available, the age groups 26 to 40 show the maximum incidence. These are, of course, ages at the time of admission to hospital.

To determine the time of onset of illness was also not considered feasible because of the unreliability of the statements as to the duration of illness. As the disease shows periodicity, the hospital class of patient either only remembers the duration of the last attack or, in order to emphasize the severity of the illness, gives an exaggerated account of the duration. In either case the data are of no value.

4. *The sex incidence.*—From the data communicated in this paper, from all the centres surveyed, it is evident that there is a very great disparity in the incidence of this disease between the sexes. Of the 3,470 cases of gastro-duodenal ulcer including cases operated and not operated on, 3,284 were males and 186 females, i.e. 18 males to 1 female. Population figures in all these areas surveyed do not show any numerical disparity in sexes, and, with the exception of purdah women amongst Mohammedans, women readily attend hospital for medical relief. At the Missionary Medical College Hospital for Women at Vellore, North Arcot district, a statistical study showed that of the 16,470 admissions, excluding maternity cases, for the years 1932 to 1938, there were only 56 cases of gastro-duodenal ulcer, i.e. about 0·03 per cent of total admissions to the hospital. From the above statistics it is evident that gastro-duodenal ulcer is very rare amongst women.

5. *Occupation and economic state.*—The present statistics bear out very conclusively that peptic ulcer in South India is a disease of the poor. It affects the poor, in whatever walk of life they are situated. Since the majority of the poor are labourers and cultivators, the largest number of cases occurred amongst them, irrespective of the race, caste or religion. In order to substantiate this observation, it may be mentioned that: (*i*) in Mysore, for three years, not one case sought admission for this disease to the paying patients' wards at the Victoria Hospital, Bangalore, (*ii*) at the Lakshmi Memorial Clinic, Madras—a private nursing home conducted by a very competent team of surgeons—a study of 1,413 case records collected over a period of ten years (1929 to 1938)—showed 19 cases operated on, of which four were poor and included the menials of the staff of the clinic, and the remaining 15 were cases of duodenal ulcer. These were amongst lawyers, merchants, landlords and clerks. These cases hailed from all over South India and were the only cases that could be considered as having occurred amongst the well-to-do.

It is argued that this class of individual gets treated medically in earlier stages of the disease. Moynihan (1927), talking of the medical treatment for gastro-duodenal ulcer, quoted a 'very distinguished surgeon' who stated that 'he rarely operates upon a patient whose ulcer has not been "cured" nine times'. Similar is the experience in South India amongst the hospital class of patients who as Bradfield (1927) pointed out 'travelled from clinic to clinic swallowing bottle after bottle of bismuth or alkaline mixture without obtaining relief from his chronic malady'. In the medical wards of hospitals and private nursing homes cases

from amongst the better class of people are not seen. It may thus be presumed that peptic ulcer is a very rare condition amongst the well-to-do in South India.

6. *Diet.*—From an account of the produce from the land in each area surveyed, it is apparent that rice is the staple food of the South Indian labouring class. The Tamilians consume it more than others, who supplement smaller quantities of rice with raghi, as is the practice amongst Canarese, while the supplement is raghi and cholam amongst the Andhras and tapioca amongst the Malayalees. The majority of the people are nominally non-vegetarians but seldom get meat or fish to eat and are, for practical purposes, vegetarians. The richer classes eat similar food, supplemented with ghee, vegetables, plantains and more generous quantities of meat, etc. Chillies and condiments are used particularly in excess by the Andhras and coco-nut products by the Malayalees. Generally speaking, milk and milk products do not form any part of the diet of the South Indian. It may here be mentioned, however, that the Brahmin, whatever be the locality to which he belongs, is a strict vegetarian inasmuch as he strictly avoids eggs, fish and meat, but his diet consisting of the staple food rice is supplemented with generous quantities of milk, butter-milk and other milk products, vegetables and fresh fruits.

Conclusions.

1. Gastro-duodenal ulcer occurs all over South India.

2. The commonest variety is an ulcer in the wall of the first part of the duodenum, which is 30 times more common than ulcer in the lesser curvature of the stomach.

3. It is almost entirely a disease of the poor agriculturist and labouring classes, amongst whom all castes and creeds are affected in proportion to their population. It rarely occurs amongst the well-to-do.

4. The disease is rare amongst women, the case incidence being 1 woman to 18 men, although the sexes are almost equal in numbers in the general population.

5. The maximum age incidence is in the age group 26 to 40 years in both sexes.

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Ind. Jour. Med. Res., **28**, 2, October, 1940.

THE EFFECT OF THE AUTONOMIC NERVES ON THE BACKWARD FLOW OF THE PERFUSING FLUID IN THE SPLEEN OF THE DOG.*

BY

M. A. BASIR, M.B., B.S., Ph.D. (Lond.),
Central Institute of Physiology, Medical College, Madras.

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THE general course of the blood vessels of the spleen and the minute structure of the sheaths of Schweigger-Seidel or the ellipsoids, were described by me in a previous paper (Basir, 1932). These observations were confirmed recently by Othmar Solnitzky (1937).

Schiff (1867) was the first to demonstrate the contraction of the spleen on stimulation of the splanchnic nerves. Roy (1881) confirmed these results and also obtained contraction of the organ on stimulation of the sensory nerves in general. Schaefer and Moore (1896) demonstrated in the splanchnic nerve both the inhibitory and motor fibres to the spleen. Recently, Barcroft and Nisimaru (1932) and Barcroft, Nisimaru and Puri (1932) obtained varying results on stimulation of the splanchnics and the vagus nerves. Again Bacq and Fredericq (1935) could not obtain any definite results by the use of drugs.

These discrepancies by different observers may be partly due to the variability of the depressor reflex, dependent on the technique adopted by them, and also partly due to the state of the blood pressure of the animal at the time of their experiments.

The following perfusing fluids were used :—

1. Fully oxygenated Ringer-Locke's fluid.
2. Fully oxygenated Dale's fluid containing eserine (1 in 100,000) and acecholine (L'Lemalte and Boinet, Paris) 0·5 mg.

* Paper read before the 27*th Indian Science Congress*, Madras, January 1940.

3. Fully oxygenated Dale's fluid containing adrenaline (P. D.) 0·5 c.c. of 1 in 1,000 solution.

Method.

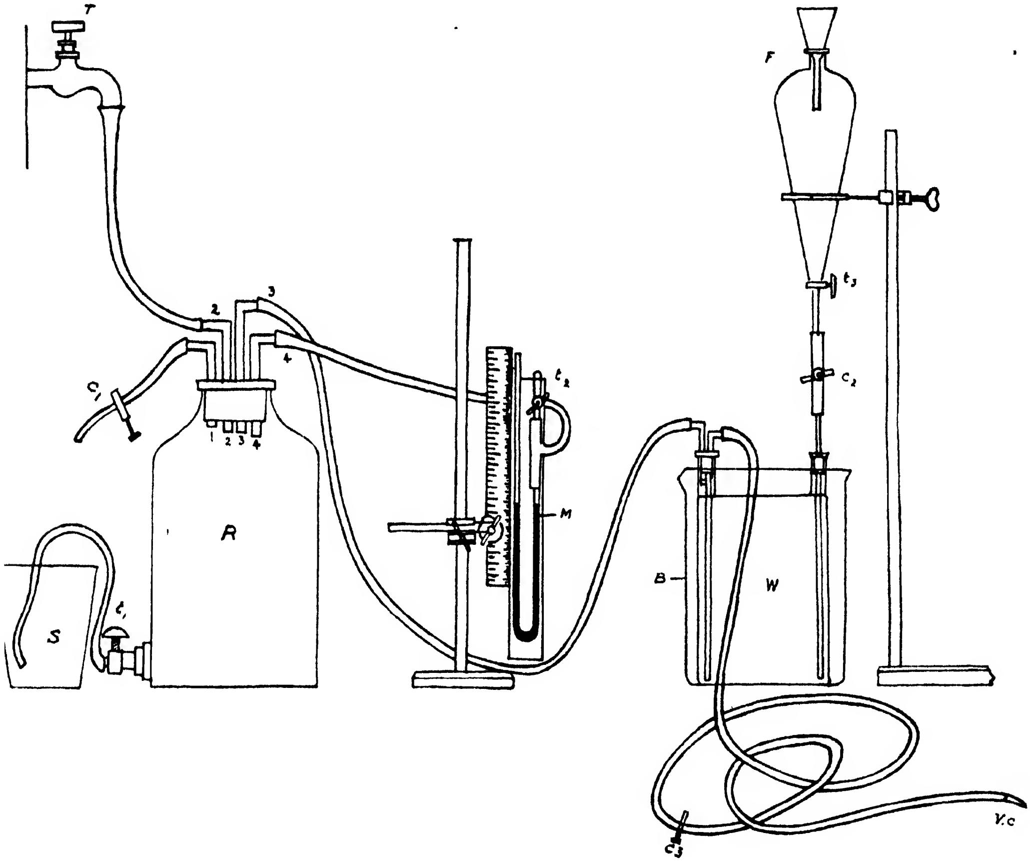
The experiments were carried out on dogs under the influence of chloralose (0·18 g./kg.) and ether. The spleen was exposed by a median incision and another incision at right angles to it on the left side below the costal margin. Then all the surrounding vessels except the splenic artery and the vein were ligatured. According to de Boer and Caroll (1924) this method eliminates any compression or twisting of the splenic vessels. A cannula was then tied in the superior mesenteric vein beyond its union with the splenic vein. An arterial cannula was similarly tied in the splenic artery close to its origin from the aorta. Nerves were carefully avoided when the various vessels were ligatured. With the cannulæ in position the wound was covered up with a cloth wetted with saline.

The arterial and venous cannulæ were then connected to a T-shaped glass-tube which in turn was connected to a Y-shaped tube. The vertical limb of the T tube carried a rubber-tubing with a clip attached to it, which acted as an outflow tube. The vertical limb of the Y tube was connected to a specially devised apparatus as shown in the Text-figure. The spleen was then perfused with the fluid either through its artery or the vein, at any desired pressure. During the experiments care was taken to avoid air bubbles and the temperature of the perfusing fluids was kept constant at 40°C.

The water from the tap (T) flows through the tube (2) into the air-tight reservoir (R) and raises the pressure within. This pressure may be measured by the manometer (M) which is connected to the reservoir (R) through the tube (4). The tube (3) transmits the same pressure to the Wolff's bottle (W) containing the fluid, for the perfusion of the spleen. The pressure with which the perfusion is to be done may also be modified by the tap (t_2) attached to one of the limbs of the manometer (M). On releasing the clip (C_3) the fluid from the Wolff's bottle (W) will flow out through the venous cannula (V. C.) which is tied to the superior mesenteric vein. The temperature of the fluid is kept at 40°C. by immersing the Wolff's bottle (W) in a water-bath (B). The tap (t_3) and clip (C_3) will put the Wolff's bottle (W) in communication with funnel (F) through which it may be filled. The tube (1) of the reservoir (R) bearing the clip (C_1) and the tap (t_1) are for emptying the reservoir (R) and the waste water will then flow into the sink (S). It is essential that the whole apparatus is air-tight.

To begin with, the spleen was perfused from its artery in order to wash out all its blood contents. This was aided by gently massaging the spleen during the perfusion. When the outflowing perfusate was clear, the direction of the flow was reversed, so that the stream now flowed from the venous to the arterial side. Pressure of the perfusate was then gradually increased till it reached 100 mm. Hg. According to Robinson (1930) the rise of pressure on the venous side would not injure the splenic tissue because the venous pressure at times may equal the arterial

when the spleen contracts. Drops of the perfusate from the arterial side, if any, were counted carefully during three minutes' intervals. Nisimaru and Steggerda (1932) allowed three minutes' interval between each step in the rise of the venous pressure because they believed that during this interval the maximal increase of the splenic volume might have been reached.



TEXT-FIGURE.

After the pressure on the venous side reached 100 mm. Hg. the flow of the perfusate from the artery to the vein was once more re-established. This was just to show that no damage had been done to the splenic tissue. The observations are recorded in the tables below.

I. *Perfusion of the spleen with the fully oxygenated Ringer-Locke's fluid.*

OBSERVATIONS.

TABLE I.

Direction of the flow.	Pressure in mm. Hg.	Quantity of the perfusate in periods of three minutes.
A.—V. ..	100	80 c.c.
V.—A. ..	35	*Nil.*
V.—A. ..	45	*Nil.*
V.—A. ..	55	*Nil.*
V.—A. ..	65	*Nil.*
V.—A. ..	75	*Nil.*
V.—A. ..	85	*Nil.*
V.—A. ..	95	13 drops.
A.—V. ..	100	82·4 c.c.

A. = Artery. V. = Vein.

Since the condition of the dog was very satisfactory another series of readings was obtained from the same animal:—

TABLE II.

Direction of the flow.	Pressure in mm. Hg.	Quantity of the perfusate in periods of three minutes.
A.—V. ..	100	82·4 c.c.
V.—A. ..	35	*Nil.*
V.—A. ..	45	*Nil.*
V.—A. ..	55	*Nil.*
V.—A. ..	65	*Nil.*
V.—A. ..	75	*Nil.*
V.—A. ..	85	7 drops.
A.—V. ..	100	82·6 c.c.

A. = Artery. V. = Vein.

A similar experiment on another dog:—

TABLE III.

Direction of the flow.	Pressure in mm. Hg.	Quantity of the perfusate in periods of three minutes.
A.—V. ..	100	85·2 c.c.
V.—A. ..	35	*Nil.*
V.—A. ..	45	*Nil.*
V.—A. ..	55	*Nil.*
V.—A. ..	65	*Nil.*
V.—A. ..	75	*Nil.*
V.—A. ..	80	5 drops.
V.—A. ..	85	10 ,,
V.—A. ..	90	11 ,,
V.—A. ..	95	10 ,,
V.—A. ..	100	15 ,,
V.—A. ..	105	15 ,,
A.—V. ..	100	87·6 c.c.

A. = Artery. V. = Vein.

II. *Perfusion of the spleen with fully oxygenated Dale's fluid and eserine* (1 in 100,000).—During the reversed flow of the fluid (i.e. from the vein to artery) acecholine 0·5 mg. was injected into the tube leading to the splenic vein. After five minutes, injected 0·5 c.c. of adrenaline (1 : 1,000) into the same tube. After a

further five minutes, injected again acecholine as before. The results are noted below.

Whenever acecholine was injected there was an increase in the volume of the spleen and after the injection of adrenaline there was a decrease. Apart from a fall in the volume of the spleen, the colour became paler and the appearance granular.

TABLE IV.

Nature of fluid.	Direction of flow.	Pressure in mm. Hg.	Quantity of the perfusate in periods of three minutes.
Dale's fluid ..	A.—V.	100	246·9 c.c.
Do. ..	V.—A.	100	135 drops.
Dale's fluid + eserine + acecholine.	V.—A.	100	342 ,,
Dale's fluid containing adrenaline.	V.—A.	100	46 drops. Towards the end of the period the outflow completely stopped.
Dale's fluid + eserine + acecholine.	V.—A.	100	133 drops. After 15 minutes the outflow increased to 336 drops per minute.

A. = Artery. V. = Vein.

III. *Perfusion of the spleen with fully oxygenated Ringer-Locke's fluid.*—The effects of stimulation of the right vagus and the right splanchnic major, from the abdominal aspect, are recorded below. The right nerves were selected because Schaefer and Moore (*loc. cit.*) believe that the nerves on the right side are more effective than those on the left.

Both the nerves were not severed but were simply slipped over the shield electrodes. This was done with the hope of studying the effects of stimulation of the nerves, with both the peripheral and central effects acting at the same time as under natural conditions. In either case a two-volt faradic stimulation was employed for 30 seconds.

TABLE V.

Stimulated nerve.	Direction of flow.	Pressure in mm. Hg.	Quantity of the perfusate in periods of three minutes.
Nil	A.—V.	100	294 drops.
Right vagus	A.—V.	100	282 ,,
Right splanchnic major ..	A.—V.	100	260 ,,
Nil	V.—A.	100	45 ,,
Right vagus	V.—A.	100	35 ,,
Right splanchnic major ..	A.—A.	100	12 ,,

A. = Artery. V. -- Vein.

DISCUSSION.

It is evident that in the dog's spleen when the pressure of the perfusing fluid reached 80 mm. to 95 mm. Hg. on the venous side, the backward flow of the perfusate from the arterial cannula commenced. This outflow gradually increased as the pressure on the venous side increased. Simultaneously with this, the volume of the spleen also increased. The distension of the splenic pulp opened up the blood vessels more and more and hence there was an increase in the backward flow of the perfusate from the arterial cannula.

When the perfusing fluid contained eserine and acecholine, the backward flow from the arterial cannula was nearly three times more than when the spleen was similarly perfused (from the venous to the arterial side) but with a perfusate without acecholine. The pressure of the perfusate in these experiments was kept constantly at 100 mm. Hg. So when acecholine was added to the perfusate there was a slight increase in the volume of the spleen which is probably due to the passive distension of the organ by the constant pressure on the venous side. Consequently, there is an increase in the backward flow from the arterial cannula. When the perfusate contained adrenaline there was a fall in the volume of the spleen due to the contraction of the organ and consequently there was a fall in the backward flow from the arterial cannula. So, then, acecholine causes a relaxation and adrenaline a contraction, by direct action on the plain musculature of the spleen.

The observations recorded, after faradic stimulation of the right vagus and the right splanchnic major, are not so amenable to explanation. In all these experiments the organ was perfused at a constant pressure of 100 mm. Hg. When the perfusate flowed from the arterial to the venous side, the stimulation of the vagus caused a slight fall in the outflow of the perfusate from the vein. Similarly, when the splanchnic was stimulated there was a further fall of the outflow of the

perfusate from the vein. Again when the perfusate was flowing backwards from the venous to the arterial side, stimulation of the vagus caused a fall and the stimulation of the splanchnic caused a still further fall in the backward flow of the perfusate from the arterial cannula. The volume of the spleen also decreased proportionately in both these instances. These observations can be explained if it is assumed that both these nerves cause a contraction of the plain musculature of the spleen in the dog. The splanchnics contract the plain muscle more than the vagus does. These results are in agreement with those of Roy (*loc. cit.*) and Schaefer and Moore (*loc. cit.*) who also noted a fall in the splenic volume of the dog on stimulation of the central end of the vagus.

It may be mentioned in conclusion that both the vagus and the splanchnics are constrictors of the spleen in the dog. The action of acecholine on the spleen is quite dissimilar to that of stimulation of the vagus in the dog.

Summary.

1. The backward flow for the arterial cannula begins in the dog's spleen when the pressure of the perfusate is increased to 80 mm. to 95 mm. Hg. on the venous side.

2. If the perfusate contains acecholine there is an increase (three times) in the backward flow from the arterial cannula. At the same time there is an increase in the volume of the spleen which is presumably due to the relaxation of the plain muscle of the spleen. If it contains adrenaline there is a decrease or even a complete cessation of the backward flow of the perfusate from the arterial cannula. This decrease in the volume of the spleen is presumably due to contraction of the plain muscle of the spleen.

3. On stimulation of the vagus and the splanchnic there is on both occasions a decrease in the backward flow of the perfusate from the arterial cannula. In the dog the stimulation of the vagus causes a fall in the volume of the spleen presumably by the contraction of the plain muscle of the spleen.

This investigation was carried out in the Department of Physiology, Medical College, Vizagapatam. In this connection I wish to express my thanks to the Demonstrator for technical assistance in all these animal experiments.

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ENDEMIC FLUOROSIS IN SOUTH INDIA.

THE OCCURRENCE OF FLUORIDES IN DRINKING WATER-SUPPLIES WITH A NOTE ON ATTEMPTS AT THEIR REMOVAL.

BY

RAO SAHIB T. N. S. RAGHAVACHARI, B.A.,

AND

K. VENKATARAMANAN, M.SC.

(An Inquiry under the Indian Research Fund Association).

(From the King Institute of Preventive Medicine, Guindy, Madras.)

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THE occurrence of mottled enamel and of other manifestations due to the presence of fluorides in drinking water-supplies in the Nellore district has been described by Shortt, Pandit and Raghavachari (1937).

In addition to the special survey of the water-supplies in this particular area, a routine systematic examination of all samples of water received in the laboratory, both from protected and unprotected supplies in the province, was undertaken in order to ascertain to what extent fluorides were present in drinking water-supplies in the other districts of the province.

The relationship, if any, between the fluorides and the other chemical constituents of the waters examined, on the one hand, and between the fluoride content and the geological formations in these areas on the other, was also studied. The results of these studies are recorded in this paper.

TECHNIQUE.

Samples were collected in 8-oz. glass-stoppered bottles by ourselves during our surveys, or by the local health staff in all other cases, who furnished at the

same time detailed information on the nature and depth of water sources at different seasons of the year, the population of the village, the occurrence of mottled enamel in children and of symptoms of bone involvement in human adults and in cattle.

The recognition of the importance of detecting fluoride in natural waters and in food materials is of comparatively recent origin and several methods have been advocated during the last six years. Many of the earlier methods were found to be inapplicable by later workers, owing to the interference of constituents other than fluorides normally present in the water. Different workers, using the same method, have also obtained divergent results. The method most commonly employed, as being the least liable to be affected by other chemical constituents when present in normal amounts, is that described by Sanchis (1936). This test is based on the property of fluorides of decolorizing the purple lake formed by zirconium nitrate with the sodium salt of alizarin sulphonic acid. A note on the experimental work carried out with various methods for the detection and estimation of fluorides is given in the *Appendix*. Having found that most of the methods experimented with were defective, for one reason or another, we finally decided to adopt a modification of the zirconium-alizarin method of Sanchis (*loc. cit.*). On the basis of numerous control experiments, we found it possible to dispense with the final boiling over a hot plate recommended by Sanchis, and to substitute in its place the storing of the *treated samples and their controls* for 24 hours at room temperature before matching. Identical results were obtained by the two procedures (viz. boiling on a hot plate and storing for 24 hours) in a series of over 200 samples containing varying amounts of fluorides ranging from 0·5 to 6 parts per million. Many of the samples showing 3 to 6 parts per million of fluorine were evaporated and the etching test confirmed the presence of fluorine in every case. The lanthanum acetate test was also positive in all these cases. By adopting the modified technique described above, it was possible to deal with a very large number of samples of water every day in the field during our surveys.

One thousand seven hundred and forty-seven samples of water from various sources in twenty-four districts of the province have thus been examined and the results are given in Table I.

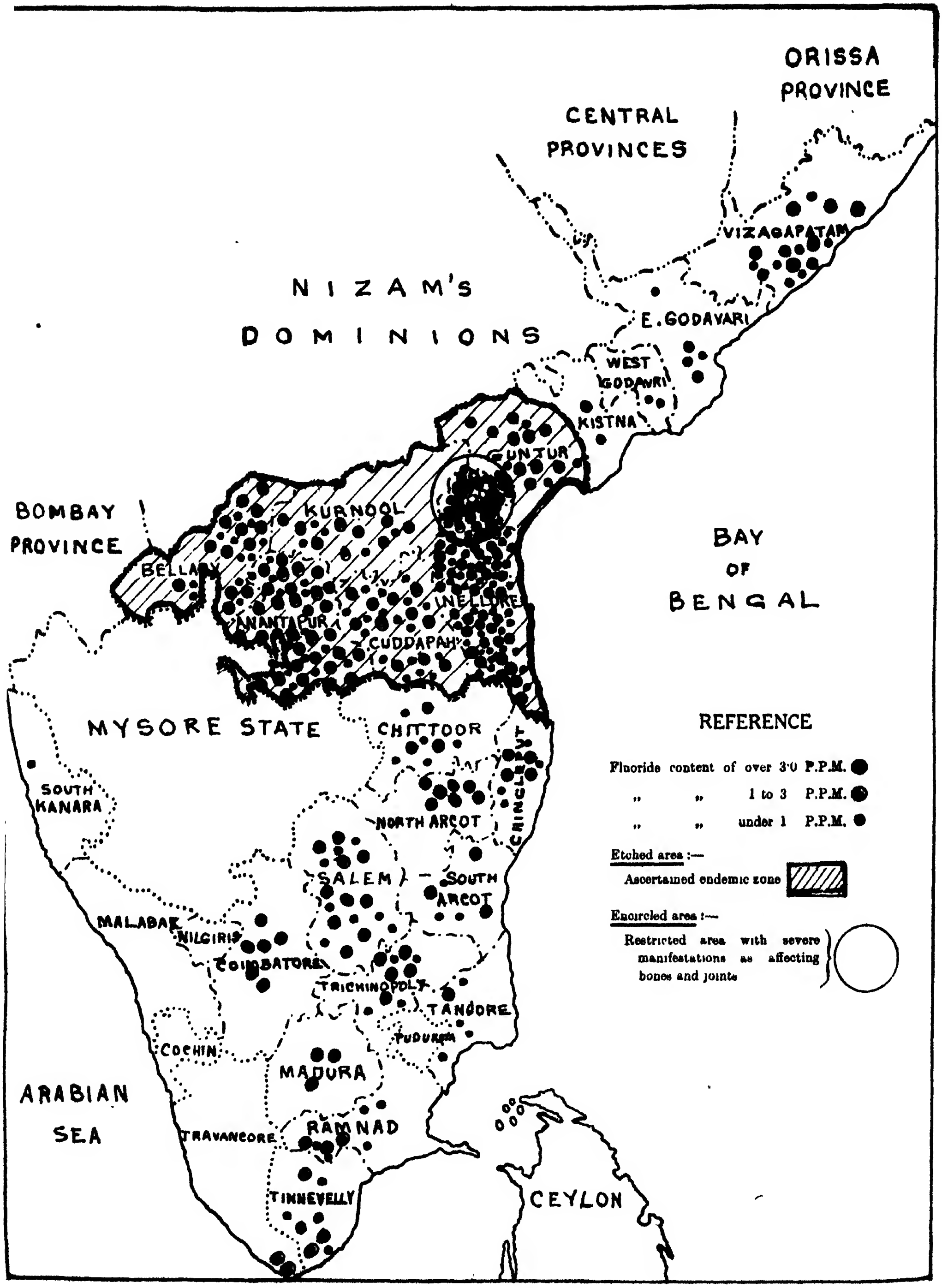
It will be seen from Table I and from Map 1 that the presence of fluorides in drinking water-supplies has been demonstrated over a very wide area in the province. Concentrations of fluorides in excess of 3 parts per million have been noted chiefly in the northern portion of the Nellore district and in a few isolated supplies in Anantapur, Bellary, Kurnool, Cuddapah and Guntur. A large number of supplies in these six districts, containing fluorides ranging from 1 to 3 parts per million, have been in actual daily use for several years. They are mostly wells laid in rocky strata to a depth of 15 to 30 feet. The fluoride-bearing waters in the other districts listed in the table and shown on the map are, however, nearly all from new bore-wells about 50 to 200 feet deep, laid in rocky strata during the last two years. These districts have hitherto relied for their drinking water-supplies on shallow wells and tanks, which did not contain fluorides but which were not considered safe from the sanitary point of view.

TABLE I.

Districts.	Fluoride content.				Total.
	Nil or trace.	Under 1 p.p.m.	1 to 3 p.p.m.	Over 3 p.p.m.	
*Anantapur ..	22	58	269	16	365
North Arcot ..	16	14	6	2	38
South Arcot ..	19	6	3	..	28
*Bellary	15	23	12	..	50
Chingleput ..	38	12	18	..	68
Chittoor	6	9	6	..	21
Coimbatore ..	8	8	2	2	20
*Cuddapah ..	15	19	32	2	68
Godavari East ..	8	3	2	..	13
Godavari West ..	18	2	..	..	20
*Guntur	8	6	18	4	36
South Kanara ..	15	1	..	..	16
Kistna	14	1	1	..	16
*Kurnool	6	9	13	3	31
Madura	15	13	8	..	36
Malabar	16	..	..	..	16
Nellore	98	106	349	58	611
Nilgiris .. .	16	..	..	..	16
Ramnad	8	4	7	..	19
Salem	11	18	22	2	53
Tanjore	46	4	1	..	51
Tinnevelly ..	12	5	3	..	20
Trichinopoly ..	18	6	7	..	31
Vizagapatam ..	29	12	16	2	59
Madras City ..	45	..	..	..	45
TOTAL ..	522	339	795	91	1,747

* The five Ceded districts referred to in the Report.

Map 1.


ORISSA PROVINCE
CENTRAL PROVINCES
VIZAGAPATAM
NIZAM's DOMINIONS
E. GODAVARI
WEST GODAVRI
KISTNA
GUNTUR
KURNOOL
BOMBAY PROVINCE
BELLARY
NELLORE
ANANTAPUR
CUDDAPAH
BAY OF BENGAL
MYSORE STATE
CHITTOOR
CHINGLEPUT
SOUTH KANARA
NORTH ARCOT
SALEM
SOUTH ARCOT
MALABAR
NILGIRIS
COIMBATORE
TRICHINOPOLY
TANJORE
PUDUKOTA
COCHIN
MADURA
ARABIAN SEA
TRAVANCORE
RAMNAD
TINNEVELLY
CEYLON
REFERENCE
Fluoride content of over 3·0 P.P.M.
" " 1 to 3 P.P.M.
" " under 1 P.P.M.
Etched area :—
Ascertained endemic zone
Encircled area :—
Restricted area with severe manifestations as affecting bones and joints

In the course of our survey in the Nellore district, it was ascertained that whole villages moved from one site to another in search of better water. In the selection of new sites, the villagers must have been guided by the brackish nature or otherwise of the water available. The examination of a limited number of samples during the early part of the investigation (1937) indicated that a high fluoride content went with high total solids. The samples of water from all sources were, therefore, tested for their chloride content, alkalinity, and pH values, while in a large number of cases a complete chemical examination was also carried out. The silica content was also estimated in certain cases. No definite correlation could, however, be established between the fluoride content and the other chemical features referred to.

It did not appear that the fluoride content had any relation to the depth of wells, for wells very close to one another but of the same depths were often found to differ widely in fluoride content.

As has already been stated, very high concentrations of fluoride were noted only in the Nellore district. Such sources, however, were not uniformly distributed over the whole district but were confined to a very limited area comprised by the three northern taluks (Podili, Darsi and Kanigiri) of the district. Table II gives the distribution of fluoride in the water-supplies of the various taluks in the Anantapur and Nellore districts, which have been surveyed in detail:—

TABLE II.

Districts and taluks.	Fluoride content.				Total.
	Nil or trace.	Under 1 p.p.m.	1 to 3 p.p.m.	Over 3 p.p.m.	
Anantapur district.					
Anantapur taluk ..	6	11	31	2	50
Dharmavaram ,, ..	1	9	45	2	57
Gooty ,, ..	5	3	19	4	31
Hindupur ,, ..	5	6	20	3	34
Kadiri ,, ..	..	1	21	..	22
Kalyandrug ,, ..	1	..	17	..	18
Madakasira ,, ..	4	9	25	3	41
Penukonda ,, ..	..	8	39	2	49
Tadpatri ,, ..	..	11	52	..	63
TOTAL ..	22	58	269	16	365

TABLE II—*concld.*

Districts and taluks.	Fluoride content.				Total.
	Nil or trace.	Under 1 p.p.m.	1 to 3 p.p.m.	Over 3 p.p.m.	
		Nellore district.			
Atmakur taluk ..	9	12	5	..	26
*Darsi " ..	8	10	42	9	69
Gudur " ..	4	5	1	..	10
Kandukur " ..	8	6	5	1	20
*Kanigiri " ..	6	11	102	21	140
Kavali " ..	7	1	7	..	15
Kovur " ..	5	1	1	..	7
Nellore " ..	14	2	..	..	16
*Podili " ..	14	33	171	27	245
Rapur " ..	7	8	7	..	22
Sulurpet " ..	8	4	2	..	14
Udayagiri " ..	1	7	5	..	13
Venkatagiri " ..	7	6	1	..	14
TOTAL ..	98	106	349	58	611

* The three northern taluks of Nellore referred to in the Report.

As stated already, surface wells in these areas are only 15 to 30 feet deep but have been laid invariably in rocky strata. During summer, the level of water falls considerably in nearly all the wells, to a foot or even less. Clay and sand are rarely met with, but *kunkur* is a common surface deposit in many of these areas. The majority of the wells in the villages yield only brackish water; only one or, in some cases, two wells in each village yield drinking water of a relatively potable quality. Water for cooking is usually obtained by the people from brackish-water wells in or near their houses.

The area comprised by Nellore and the districts already mentioned by name, considered geologically, has three distinct formations: (1) the alluvial and laterite

formation on the east coast as far west as Guntur, Ongole and Kandukur ; (2) the Cuddapah-Kurnool series represented in the Nellore district on its western border and in parts of the other three districts and (3) the archæan gneisses. The laterite and alluvial formations showed little or no fluorides in the ground waters examined. The highest concentrations of fluoride were found in the water of the wells in Darsi, Podili and Kanigiri all of which lie in the archæan gneiss area. As we proceeded westward towards and past the Velikonda hills (the Cuddapah-Kurnool formations), the fluoride content was found to diminish and to reach an average level of 1 to 1·5 parts per million, a few stray samples, however, showing 2 to 3 parts per million.

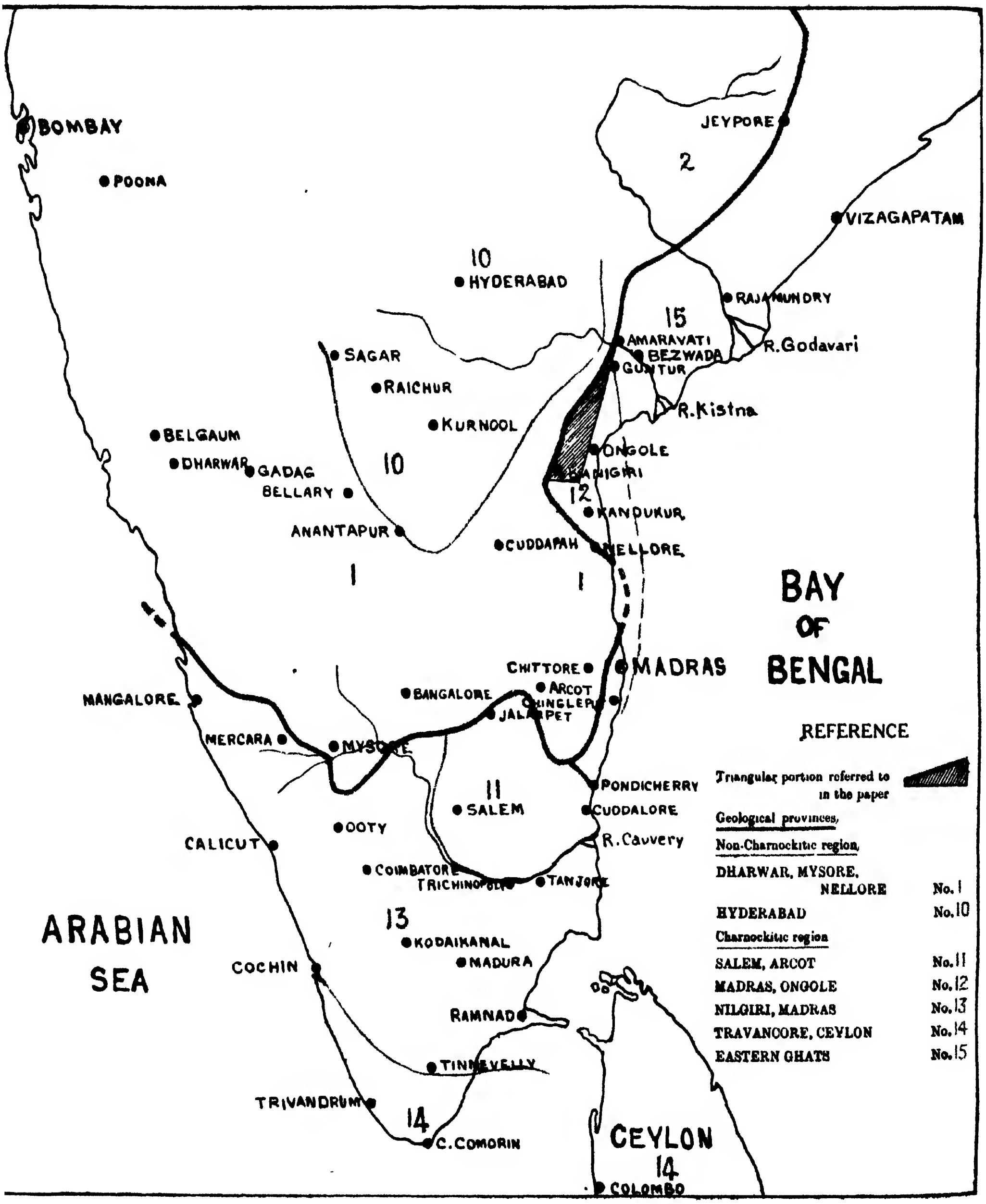
This zone lies in the geological provinces of (1) Madras-Ongole (charnockitic), (2) Dharwar-Mysore-Nellore, (3) Hyderabad (both non-charnockitic) described in Parts I and II of Vol. LXX (1936) of the *Memoirs of the Geological Survey of India*.

Judged by the occurrence of fluoride in deep ground waters in many districts from Vizagapatam in the north to Tinnevelly in the south, and from Chingleput in the east to Coimbatore in the west, it may not be incorrect to assume that fluoride is a normal constituent of the rock minerals found both in the charnockitic and non-charnockitic regions. But its presence in high toxic quantities in certain areas, e.g. northern Nellore and isolated places in Cuddapah, Anantapur and Coimbatore districts. is probably associated with the occurrence in these areas of rich fluoride-bearing rocks and also possibly of faults and fissures connecting igneous intrusions and limestone deposits with other strata.

An attempt was made to correlate this factor with the data given in the *Memoir of the Geological Survey of India* referred to above. The area showing the highest fluoride concentrations is found to lie in ' the narrow belt of granitoid rocks stretching N.N.E. from south of Kanigiri (Nellore district) to the Kistna river, near Amaravathi in Guntur district '. This area is demarcated by us by a *triangle* on the geological map of the area under survey (Map 2). This narrow belt divides the schistose gneisses or schists of this region into a western and an eastern tract ; the eastern tract and the central belt are included in the Madras-Ongole province, No. 12 of Fermor (1936). The specimens of rocks collected by us from well excavations and river beds in the central belt area have been kindly identified for us by Professor C. K. Krishnaswami Pillai, M.A., M.SC., D.I.C., of the Presidency College, Madras, and by the Geological Survey of India, and found to be rich in fluorite and fluorapatite.

There exists a popular belief among the people of the Nellore district that the micaceous nature of their water-supplies is responsible for many of their ailments. But the results of our investigation have shown that waters from areas traversed by the micaceous and talcose schists extending N.N.E., from Kaluvaya and Talagapur to Saidapuram in the Nellore district, contain only about 1 part per million of fluoride. Thus, the mica mines of Saidapuram, the mica schist areas in Maripudi, Pedda-Arikatla and Chinna-Arikatla showed a fluoride content of only 1 to 1·2 parts per million.

MAP 2.


BOMBAY
POONA
JEYPORE
2
VIZAGAPATAM
10
HYDERABAD
RAJAHMUNDRY
15
AMARAVATI
BEZWADA
GUNTUR
R.Godavari
SAGAR
RAICHUR
KURNOOL
R.Kistna
BELGAUM
DHARWAR
GADAG
ONGOLE
10
KANIGIRI
BELLARY
12
KANDUKUR
ANANTAPUR
CUDDAPAH
NELLORE
1
1
BAY OF BENGAL
CHITTORE
MADRAS
BANGALORE
ARCOT
CHINGLEPUT
MANGALORE
JALARPET
MERCARA
MYSORE
REFERENCE
Triangular portion referred to in the paper
11
PONDICHERRY
SALEM
CUDDALORE
OOTY
Geological provinces,
Non-Charnockitic region,
CALICUT
R.Cauvery
COIMBATORE
TRICHINOPOLY
TANJORE
DHARWAR, MYSORE, NELLORE No. 1
HYDERABAD No. 10
13
Charnockitic region
ARABIAN SEA
KODAIKANAL
COCHIN
MADURA
SALEM, ARCOT No. 11
MADRAS, ONGOLE No. 12
NILGIRI, MADRAS No. 13
TRAVANCORE, CEYLON No. 14
EASTERN GHATS No. 15
RAMNAD
TINNEVELLY
TRIVANDRUM
14
C.COMORIN
CEYLON
14
COLOMBO

Fluoride removal.

Ever since the presence of fluorides in water-supplies came to be specifically incriminated, about ten years ago, as the causal agent in the production of mottled enamel, several methods have been tried for effecting the removal of fluorides from drinking water-supplies in America. Deep sources containing fluorides in toxic amounts have been abandoned in favour of shallow sources. Where these latter were, however, not available, methods of removal have been and are being tried and these fall under two heads—

(*i*) those involving the precipitation of the fluoride ions by chemicals like alum, lime and magnesia, followed by subsequent filtration, and

(*ii*) those involving the passage of the water through filters containing contact materials of varying compositions.

The first of these two methods is expensive both in initial and in recurring costs and will not, therefore, suit rural conditions obtaining in this province. It may have a limited field of application in larger towns.

Boruff (1934), McKee and Johnston (1934) and Scott *et al.* (1937) found that very large doses of alum were required to effect a satisfactory removal of fluoride. Thus, a dose of 119 grains of alum per gallon was necessary to reduce the fluoride content from 5 to 1 part per million. Our own experiments have confirmed these findings. A reduction in the fluoride content of waters is reported to occur in the process of lime-softening. In our experience, the addition of 5 to 30 grains of freshly-slaked lime to a gallon of river-water artificially made to contain 5 parts per million of fluoride, caused no significant reduction.

Scott *et al.* (*loc. cit.*) have shown that the degree of fluoride removal by lime is a function of the removal of magnesium, the reduction being approximately equal to 7 per cent of the initial fluoride multiplied by the square root of the magnesium removed. They suggest, therefore, that magnesium be added to the water if necessary, enough lime being added afterwards to raise the pH to 10·5. The treated water has finally to be re-carbonated to bring the pH down to 7·4. This method is neither sufficiently practical nor economic for general application in our rural areas.

Elvove (1937) found that when a fluoride-bearing water was agitated by means of a current of air with a small quantity of calcined magnesite, satisfactory removal took place. As this method would be impracticable under our local conditions, a variation in the method was tried by us. Commercial grades of '*granular*' magnesium carbonate and magnesite (kindly supplied by the Salem Magnesite Works) were put up in glass-tube filters and water containing 5 parts per million of sodium fluoride was passed through. The pH of the water increased from 7·9 to 8·4 and the removal in three successive lots of 250 c.c. samples was 62, 50 and 58 per cent, respectively. Toxic amounts of fluoride (1·9 parts per million) filtered through, even in the first lot of 250 c.c. of water. Better removals were noticed when the samples were *shaken up* with magnesia. Thus, a tap-water with added fluoride of 4·4 parts per million (of sodium fluoride) was brought down by agitation

to a concentration of 0·7 part per million. Similarly, a sample of natural water from Podili with a fluoride content of 6 parts per million was reduced to 1 part per million.

McKee and Johnston (*loc. cit.*) found activated carbon useful and effective but only at a pH value of less than 3·0. Our own experiments with granular activated Jewlcarbon showed very satisfactory removal but only when the pH was kept considerably below 3. This method, being very expensive, has to be ruled out.

Filters of broken marble and Cuddapah stones were tried and found to effect little or no reduction in the fluoride at the normal pH of the water, viz. 7·4. If the water was, however, carbonated to bring its pH to the neighbourhood of 5 the fluoride was reduced from 1·8 to 1·3 parts per million. This method is not, therefore, of any real practical value.

Kramer (1934) found that a filter containing a mixture of sand and powdered aluminium removed fluorides effectively. Our experiments showed that a pound of the powder (local Madras price Re. 1-4-0) effected a satisfactory removal of fluorides from only 1·2 gallons of water containing 5 parts per million. Parallel experiments, using the powder alone and in combination with sand showed that better results could be obtained with the latter.

Our experiments on the removal of fluoride from water by storing it in aluminium vessels for 24 hours showed that the fluoride was reduced from 5 parts per million to about 3 parts per million by such storage.

An aluminium zeolite was especially made for us by the Jewell Filter Co., London, as also a copper zeolite. Both of these effected very satisfactory removal at first but rapidly became exhausted. The most serious disadvantage with these two materials was that aluminium and copper appeared in large quantities in the filtrates.

Among other substances suggested for use as exchange materials for the removal of fluorides are activated alumina (Churchill, 1937), tricalcium phosphate (Adler, Klein and Lindsay, 1938), granulated bone (Smith, 1937) and synthetic resins (Adams and Holmes, 1935). Our experiments with a synthetic resin made from meta-phenylene-diamine did not yield satisfactory results.

We have since carried out a large number of experiments using '*Defluorite*', a proprietary product obtained for us by Messrs. Jewell Filter Co., Calcutta. This material, which has about 90 per cent alumina in its composition, was supported in our experiments over a layer of 1 inch washed sand and 2 inches broken stone in a glass-tube filter, 18 inches long and 1½ inches diameter. Tap-water with definite amounts of sodium fluoride added to it was passed through this filter at the rate of about 0·5 gallon per hour.

In the first experiment, water containing 5 parts per million of fluoride was passed through 100 g. of the '*Defluorite*'. The fluoride content was reduced to zero and the efficiency of the material remained unimpaired till after 26 litres had passed through. In the next experiment, 20 g. of the material and water

containing 10 parts per million of fluoride were used. Traces of fluoride appeared when 7·5 litres had passed through. One part per million of fluoride appeared when 9 litres had been passed. But, it is our considered opinion that in bulk filtration, 14 litres could be passed through before the effluent would have built up a toxic amount of fluoride. In other words, one pound of the material will effect satisfactory removal of fluoride from about 230 gallons of a water containing 3 parts per million. On the plant scale, where much higher rates of filtration are usually attained, even better results might be expected. Regeneration with an 8 per cent solution of sodium hydroxide followed by treatment with 0·5 per cent hydrochloric acid was found to revive the efficiency of the material. The regeneration was not, however, sustained as toxic amounts appeared in the effluent again after the passage of only 4 litres of water.

Summary.

The occurrence of fluorides in drinking waters with resulting mottled enamel in children in a more extensive area than that originally described, viz. Nellore district, is recorded. This area comprises the districts of Bellary, Kurnool, Anantapur, Cuddapah, and Guntur.

Fluorides, in small or moderate amounts, have been detected also in stray samples of deep ground waters from Chittoor, North Arcot, Chingleput, Madura, Coimbatore, Ramnad, Salem, Trichinopoly, Tinnevelly and Vizagapatam districts.

Specimens of rock from the severely affected areas of the Nellore district were found to contain fluorite and fluorapatite in fairly large amounts.

Various methods for the estimation of fluorides in water have been tested. A modification of the method of Sanchis is described which was found to be the most suitable consistent with accuracy and ease of performance under field conditions.

Several methods for the removal of fluorides from water have been experimented with but no chemical process suitable for use under conditions obtaining in the rural areas of our province has yet been evolved. '*Defluorite*' (a proprietary material) would appear, however, to be the most promising but the economics of this method will require further careful investigation.

No correlation has been found between the concentration of fluoride in water and the depths of wells on the one hand and between the fluorides and other main chemical features of the waters, such as chlorides, silica, alkalinity and pH values on the other.

The fluoride concentration and the incidene of fluorosis would, however, appear to correlate in a general way with the broad geological divisions comprised in the area.

As indicated in a previous paper by Shortt, Pandit and Raghavachari (*loc. cit.*), the severity of the intoxication from fluoride in north Nellore would appear to bear a definite relation to the concentration of fluorides in the water-supplies used for

drinking and cooking. Cases of fluorine intoxication marked by severe bone involvement have hitherto been recorded only among workmen exposed to massive doses of fluorine derived from cryolite factories (Roholm, 1937). The factors operating to produce the more severe manifestations of fluorine intoxication which is associated in this area with high fluoride concentrations in ground water-supplies, will be discussed in another paper.

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APPENDIX.

THE DETECTION AND ESTIMATION OF FLUORIDES.

The recognition of the importance of detecting and estimating fluorine in natural waters and in other materials pertaining to the animal and vegetable kingdom is of comparatively recent origin. Several methods reported to be quite suitable by some have been shown by subsequent workers to be fallacious and liable to interference by other chemical constituents normally present in natural waters.

Very few comparative studies would appear to have been made in this direction. It was somewhat difficult, therefore, to choose the most reliable method which would also be suitable alike for field and laboratory work.

Thus, the test of Hagen (1934) in which as little as 0·5γ of fluorine was said to cause the formation of oily droplets when the sample was warmed with concentrated sulphuric acid in a test-tube, the walls of which were coated with a mixture of potassium dichromate and sulphuric acid, was found to be defective. Pertussi (1934) claimed that as little as 0·00004 g. hydrofluoric acid gave a perceptible precipitate when the solution was heated with a mixture of mercury succinimide and benzidine solutions. Tests were conducted by us using amounts of fluorine ranging from 0·01 mg. to 0·20 mg. in Widal tubes and the presence of the precipitate was looked for with a hand lens. The precipitate appeared only after a considerable time and even then required vigorous scratching. Many samples of water which did not contain any fluorine also yielded precipitates with this reagent. Mayer and Schulz's (1925) method of precipitating the fluoride as lanthanum acetofluoride in the presence of eosin was found to be satisfactory but unsuitable for use in the field. Friegl and Krumholz (1929) advocated the volatilization of fluorine as silicon tetrafluoride by heating with concentrated sulphuric acid and sand, absorbing the gases in water and testing the aqueous solution for silica with ammonium molybdate and benzidine. This test was found to be quite sensitive. It is, however, liable to give false positives as a result of the solution of silica from glass receivers (*cf.* Bowes and Murray, 1935).

Spectrographic methods (Churchill, 1931; Petrey, 1934) have been used and fluorides detected in a band spectrum of calcium fluoride which appeared when a substance containing calcium and fluorine is excited in an electric arc. If the residue contained sodium in excess, calcium would have to be externally supplied in this test.

The etching of glass by the action of the vapours obtained by heating the fluorides with concentrated sulphuric acid is the most specific of all the qualitative tests but is obviously unsuited for field work. We obtained very satisfactory results by using a special apparatus which consisted of an ordinary lead crucible provided with a heavy lid of the same metal. The top portion of the lid was so

grooved as to take a microscope slide. In the centre of the lid was a small hole which permitted all the fumes of hydrofluoric acid to act only on a particular spot on the glass surface. Attempts to convert this test into a semi-quantitative test by carrying it out under identical conditions were not, however, successful.

The methods used in the estimation of fluorides are gravimetric, volumetric or colorimetric. Gravimetric methods involve the precipitation of fluorides as calcium fluoride, lead chlorofluoride or triphenyl-tin-fluoride. In the volumetric methods, the fluorine is titrated against salts of the rare earth metals (e.g. cerium or thorium) or against salts of aluminium and iron. Colorimetric methods are based on the property of fluorides of bleaching the purple colour of the lake formed by zirconium salts with alizarin-S or purpurin or of the yellow colour formed by a titanium salt with hydrogen peroxide or on its property of inhibiting the development of the red colour formed by ferric salts with thiocyanates (Foster, 1933).

Precipitation of fluorides as lead chlorofluoride followed by the decomposition of the washed precipitate and subsequent estimation of the liberated chlorides gave reproducible results when at least 10 mg. of fluorides were present in the sample. Attempts to adapt this method for micro-estimation of fluorine by carrying out the test in 15 c.c. centrifuge tubes and separating the precipitate by centrifugalization proved unsuccessful.

Kurtenacker and Jurenka (1930) titrated fluorides against salts of aluminium or cerium using methyl-red as internal indicator. Working with an N/6 solution of aluminium chloride they obtained satisfactory results with about 0·6 g. of fluorine. When more dilute solutions were used (N/60 and N/600) the end-points became vague and indiscernible. The use of cerium salts suffers from the additional disadvantage that even small quantities of sulphates are liable to induce serious errors. Willard and Winter (1933) titrated fluorides against a standard solution of thorium nitrate using the purple lake formed by zirconium nitrate and alizarin-S, as the internal indicator. This method has since been studied by many other workers. Improvements have also been suggested. But all these three methods are liable to interference by salts of the alkaline earth metals, aluminium, iron and by sulphates and phosphates. Since the interfering substances are normally present in nearly all natural waters, these methods would be applicable only when the fluorine has been isolated from the other ingredients by volatilization.

When a large number of natural waters has to be examined every day, the removal of the interfering materials by distillation becomes impracticable. We have, therefore, to search for a rough and ready method which can be easily applied in the laboratory as well as in the field, and which is not affected by the interfering ions when present in the average concentrations in which they are normally found in potable waters. The colorimetric methods are obviously, therefore, the most suitable for the purpose.

The method advocated by Foster (*loc. cit.*), in which the amount of fluorine is measured by the inhibition of the development of the red colour when known amounts of a ferric salt and of thiocyanate are added to the sample, is subject to the limitation that a variety of substances normally present in natural waters can

interfere with the test. The colour shades are difficult to read and the colours fade away somewhat rapidly.

The method suggested by Merwin (1909) and modified by Sharples and McCollum (1933) was based on the property of fluorides of inhibiting the development of the yellow colour produced when a solution of a titanium salt is treated with hydrogen peroxide. Bowes and Murray (*loc. cit.*), however, found that this method was not sufficiently sensitive in detecting the smaller ranges of variation in the fluoride content. The conditions governing this bleaching effect were investigated in detail by Wichman and Dahle (1934) who stressed the importance of accurate adjustment of the pH to 1·5. This requirement was not easy to attain in practice, particularly on the field. Comparisons of colour in Nessler glasses are difficult and a photometer would be necessary to obtain satisfactory results. In addition to these limitations, it would be necessary to take an aliquot containing about 0·05 mg. of fluorine for the tests. In doing so, the error in comparison of the colours is liable to be multiplied nearly a hundredfold when a water containing 5 parts per million of fluoride is under test.

De Boer (1925) reported that the purple lake formed by zirconium nitrate with alizarin-S, was bleached by fluorides and that the sensitivity of the reaction could be increased by the presence of an acid in the medium. Barr and Thorogood (1934) tried the addition of acid and, using zirconium oxychloride, worked out the optimum ratios of hydrochloric acid required for the best sensitivity. They advocated the use of 4 c.c. of 3 N hydrochloric acid for each 100 c.c. of the sample. While the addition of hydrochloric acid prevented the interference of aluminium, arsenates, phosphates, borates, silicates and oxalates, it did not eliminate interference by sulphates. Elvove (1933) suggested the use of standards with synthetic water which had the same concentration of sulphates as the samples under test. Thompson and Taylor (1933) used this method for the estimation of fluorides in sea-water. Their standards were adjusted to contain the same amount of chlorides as the samples under test.

Sanchis (*loc. cit.*) found that by adding to the samples and to the standards 2 c.c. of 3 N sulphuric acid and 2 c.c. of 3 N hydrochloric acid instead of 4 c.c. of 3 N hydrochloric acid, the interference due to the sulphates could be eliminated. The method of Sanchis consists in treating 100 c.c. of the sample (filtered if necessary) and 100 c.c. each of the standards containing 0, 0·25, 0·5, 0·75, 1, 1·5, 2, 2·5 and 3 parts per million of added fluoride with 2 c.c. of 3 N hydrochloric acid and 2 c.c. of 3 N sulphuric acid and then adding 2 c.c. of an indicator solution to each. The stock solution of the indicator is prepared by mixing a 0·87 per cent solution of zirconium nitrate with an equal volume of a 0·17 per cent solution of alizarin-S. This solution is diluted five times before use. The samples and standards are kept on a hot plate and quickly brought to the boil. They are then left over for four hours or more when the colours are compared. Sanchis claimed that chlorides, sulphates, bicarbonates, sodium, calcium and magnesium up to 500 parts per million, manganese up to 200 parts per million, silicates up to 50 parts per million, phosphates, boron, copper and iron up to 5 parts per million did not interfere with the accuracy of this test.

The process of boiling over a hot plate suggested by Sanchis appeared to us to be unnecessary. In the absence of a set of flasks of uniform thickness and size and of a large hot plate—facilities not easily procurable in the field—there would be a lack of uniformity in heating. Experiments were conducted, therefore, to see if the boiling could be dispensed with. A large number of samples of natural waters containing fluoride in varying amounts and of fluoride-free waters to which known amounts of sodium fluoride or sodium silico-fluoride were added was examined by us using the method of Sanchis. A duplicate series of the same samples was similarly treated but instead of boiling over a hot plate, the flasks were left at room temperature in a closed box for 24 hours. Appropriate standards were run in the same way—one set with and the other without boiling. Though the degree of bleaching for the same concentration of fluoride was greater in the tubes whose contents had been boiled, the results obtained by comparison with the appropriate standards in the two series were the same. We have, therefore, dispensed with boiling since October 1937.

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ENDEMIC FLUOROSIS IN SOUTH INDIA.

A STUDY OF THE FACTORS INVOLVED IN THE PRODUCTION OF MOTTLED ENAMEL IN CHILDREN AND SEVERE BONE MANIFESTATIONS IN ADULTS.

BY

C. G. PANDIT, M.B., B.S., Ph.D., D.P.H., D.T.M.,

RAO SAHIB T. N. S. RAGHAVACHARI, B.A.,

RAO SAHIB D. SUBBA RAO, M.B., B.S., B.S.SC.,

AND

V. KRISHNAMURTI, M.B., B.S.

(*An Inquiry under the Indian Research Fund Association.*)

(*From the King Institute of Preventive Medicine, Guindy, Madras.*)

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THE discovery of an area in Madras Province showing a high degree of chronic intoxication due to the presence of fluorides in drinking water was reported by Shortt *et al.* (1937*a*). The general clinical picture, as revealed by a study of several cases in the affected area and by a detailed study of ten cases admitted into the General Hospital at Madras, was described by Shortt *et al.* (1937*b*). The distribution of fluorides in water in the province based on a systematic study of samples derived from several protected and unprotected supplies has been recorded by Raghavachari and Venkataramanan (1940).

These studies have shown that the problem of endemic fluorosis in South India is two-fold: firstly, the prevalence of a dental dystrophy in children, commonly known as 'mottled enamel', over a comparatively wide area, and, secondly, the occurrence, in a restricted area, of severe manifestations in adults of chronic fluorine intoxication involving the spine, joints and ligaments to which the name of endemic fluorosis has been given. So far as we know 'in no previous description has the disease been so directly attributable to naturally existing surface or near

surface water-supplies, so severe in its manifestations in later life, as distinct from its effects on the teeth of children, and on so large a scale' (Shortt *et al.*, 1937*a*).

As has already been indicated, the majority of the drinking water-supplies in actual use for several years in the Nellore and in the Ceded districts showed a fluoride content ranging from 1 to 3 parts per million. Only a comparatively small number of sources contained over 3 parts per million and the maximum content recorded has never exceeded 6 parts per million. While the almost universal occurrence of mottled enamel in children is, therefore, to be expected in such areas, the prevalence of the more severe forms of intoxication is less readily understood since such manifestations have not been recorded in any of the highly endemic areas in other parts of the world where the drinking water-supplies contain over 6 parts per million, such as Maldon in England, or even as much as even 18 to 20 parts per million as in Texas, U. S. A.

The condition as described by Shortt *et al.* (1937*b*) was analogous to that recorded by Roholm (1937) amongst cryolite workers in Denmark, as evidenced not only by the similarity of the clinical symptoms but also by the radiological findings in both cases. But, as pointed out by Shortt *et al.* (1937*a*), this was the first instance where the condition was traceable to fluorides present in drinking water, in the comparatively small amounts noted above. It, therefore, became apparent that one or more factors peculiar to these areas, in addition to the presence of fluorides in the drinking water were probably involved in bringing about this condition.

A detailed investigation was, therefore, made in several selected areas of the Nellore and adjoining districts to ascertain what these factors were and how they operated. The results of this investigation are recorded in this paper.

General incidence of mottled enamel in the province.

The general distribution of fluoride-bearing waters in the province has already been recorded (Raghavachari and Venkataramanan, *loc. cit.*). These areas fall into two groups: (*a*) those where the fluoride-bearing waters have been in actual daily use for many years (as in Nellore and the five Ceded districts) and (*b*) those which have only recently been brought into use.

As was to be expected, the former areas alone showed the almost universal prevalence of mottled enamel in children, in varying degrees of severity. With the exception of the coastal alluvial and lateritic tract of the Nellore district, which is free from fluorides, the other parts of this district and the whole of the Anantapur district showed an incidence of from 50 to 90 per cent of mottled enamel.

The presence of famine conditions in the Bellary district during the summer of 1938, and the consequent opening of large relief camps there, provided an opportunity for an extensive survey of a large number of the inhabitants of that district drawn from several taluks. Thus, a total of nearly fifty thousand inhabitants of both sexes and of various age-groups was readily available for examination. The

incidence of mottled enamel in children was found to be 70 per cent. The samples of water examined from the villages in Bellary showed the presence of fluorides varying from 0·6 to 2 parts per million.

A survey of twenty-one villages in the Cuddapah district revealed the presence of mottled enamel to the extent of 60 per cent and more, the severity depending, as in other districts, on the concentration of the fluorides in water-supplies in actual use. In the course of a survey of mottled enamel in school children in Anantapur district, it was found that all the children from Pattikonda in Kurnool district who were attending a school in Tadpatri (Anantapur district) showed the dental dystrophy. Dr. Aykroyd's recent observations in the Kurnool district also support this finding. The incidence of mottled enamel in this district has not yet been fully investigated, but it is likely to be more or less similar to the other Ceded districts.

General incidence of chronic fluorine intoxication in the province.

The preliminary survey carried out in 1937 revealed the presence of the more severe forms of intoxication, as affecting bones and joints in a few villages in each of the three northern taluks of Nellore district, viz. Podili, Darsi and Kanigiri. It was decided, therefore, to make an extensive survey, with the help of the local health staff, in all the villages of the three taluks to determine if the condition was widely prevalent. The health staff received specific instructions for carrying out the survey and their findings were subsequently verified by us. Thus, in Darsi taluk sixty-five out of a total of one hundred and nineteen villages were surveyed and in nineteen of them severe bone affections were noted. At least fifteen villages and hamlets out of one hundred and ninety in Podili taluk and thirty-one villages and hamlets out of nearly eight hundred in the Kanigiri taluk, were found to be similarly affected. These three taluks happen to lie at the base of the triangular zone referred to by Raghavachari and Venkataramanan (*loc. cit.*) where the fluoride content of many of the water-supplies was about 3 parts per million or over.

The villages in all the other taluks of the district were found to be almost entirely free. Two villages, however, in the Kandukur taluk situated on the borders of the Kanigiri taluk showed the presence of people with bone involvements and the fluoride content of the water in these villages was between 2 and 5 parts per million. The relation between a high fluoride concentration in water and the incidence of severe intoxication was thus readily apparent.

From the reports received from the health officers and from the results of fluoride estimations in waters, it does not appear that this form of chronic fluorine intoxication is prevalent to any large extent in the adjoining district although a critical survey might reveal its presence in a few isolated instances.

Detailed survey of selected areas in the Nellore district.

Podili and its hamlets.—During the course of the general survey in Podili, it was noticed that the incidence and severity of chronic fluorine intoxication differed

widely in different parts of the main villages and its hamlets. A detailed house-to-house survey was, therefore, carried out in this area with particular reference to mottling of the teeth in children and to affection of bones in adults. The dietetic and other habits of the people, sources of water-supply, occupations, lengths of residence and all other factors likely to have a bearing on the condition were carefully studied. The symptoms noted with regard to teeth and bone affections were classified in each case as mild, moderate and severe. It may be noted here that, on account of the general prevalence of the condition, it was by no means difficult to judge of the severity or otherwise of the cases by clinical examination alone. Otherwise, had only a few stray cases been present, such a condition could not have been diagnosed without the help of a radiological investigation.

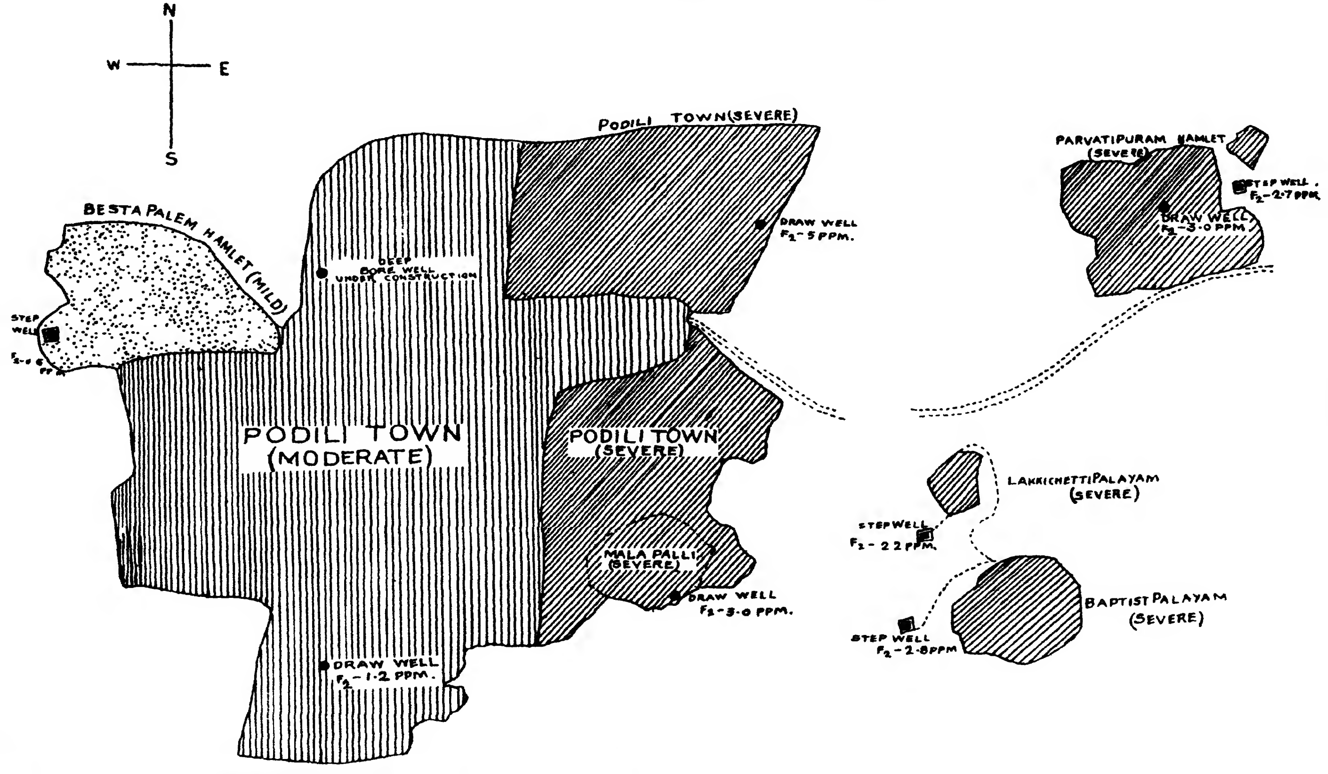
(*a*) *Water-supply.*—It was found that Podili and its environs could be divided into three areas on the basis of the fluoride content of the drinking water-supplies. The inhabitants of a localized small area in the west derived their water-supply from a well with a fluoride content of only 0·6 part per million. The central and major part of the town obtained its water from a well with a fluoride content of 1·2 parts per million, while the eastern portion obtained its water from wells which showed 3 to 5 parts per million of fluorides. The three hamlets of Podili, viz. Parvathipuram, Lakkisettipalayam and Baptistpalayam, are situated a mile and a half to the east of the main village. The fluoride content of the well-water in these hamlets was 3, 2·2 and 2·8 parts per million respectively. The relative positions of the main village and its hamlets are shown in the Map.

(*b*) *Incidence of mottled enamel in children in the area.*—The total number of children examined of the ages of 5 to 15 was 1,115 (588 boys and 527 girls). Seventy-four per cent of these showed mottled enamel, 23 per cent mild, 20 per cent moderate and 31 per cent severe. The incidence was almost the same in both sexes, 74·2 per cent in boys and 75·2 per cent in girls. The severe type of mottling was observed in 34·3 per cent of boys and 27·9 per cent of girls. The occurrence of mottled enamel in the three areas of the main village showed no variation as regards its incidence but could be correlated with the fluoride content of water as regards severity. The toxic level of fluorides in water, at which mottling of the enamel occurs, appears to be in the neighbourhood of 1 part per million. There was some evidence to show that the deciduous teeth were affected in some cases. Thus, out of 387 children below 5 years of age, 141 or 36 per cent showed a mild degree of mottling. The findings of other observers would, however, go to show that deciduous teeth are seldom, if ever, affected. Dean (1936) and Mahoney (1938), however, have recorded mottling of deciduous teeth in a few cases, in areas of medium and marked severity of this condition.

(*c*) *Incidence of chronic fluorine intoxication in adults.*—It was found that the severe manifestations of chronic fluorine intoxication appeared only in persons who had continuously resided in the area from childhood and thus exposed themselves to the risk for at least 25 years or more. The intensity of this condition has been strikingly brought out by the survey. One thousand one hundred and ninety-two adults were examined belonging to various age-groups over 21 years of age, of which 601 were men and 591 women. The degree of bone lesions in one or more

MAP OF PODILI TOWN AND ITS HAMLETS.

INCIDENCE OF FLUORINE INTOXICATION.



of following parts of the body, viz. spine, chest and joints, was used for classification into mild, moderate and severe. Three hundred and eighty-seven adults of 74·4 per cent of the total number examined, showed bone affections, the sex incidence being 75·2 per cent in men and 73·6 per cent in women. The advanced condition, characterized by a complete rigidity of the spine, causing the affected persons to turn the whole body in order to turn the head sideways, immobility of joints of both extremities, the fixation of the thoracic wall so that breathing became entirely abdominal and other symptoms during the later stages due to pressure on the spine, was noted in 13·0 per cent of men and 15·2 per cent of the women examined.

The incidence of bone affections would appear to be higher in adults who had resided for over 15 years in this area than in the group with a shorter stay. As has already been stated, severe cases were found mostly in the eastern part of Podili town and its hamlets of Parvathipuram and Baptistpalayam, the water-supplies of which showed the highest fluoride content, viz. 2·5 parts per million and over.

In the eastern part of the town is also situated a small area—Malapalli—which is occupied exclusively by Malas, i.e. sweepers. The adjoining area is inhabited by other Hindus and Mohammedans. The Malas obtain their water-supply from a well with a fluoride content of 3 parts per million and have no access to the well in the neighbourhood with a fluoride content of 1·2 parts per million used by the other inhabitants. The survey showed that the incidence of severe fluorine intoxication as described above was about 30 per cent in the Malas as compared with 12 per cent in the adjoining area.

Apart from the habits of the people, their diet, etc. the significance of which will be discussed later, the main Podili villages and Malapalli provide a significant indication of the effect of the concentration of fluorides in water on the severity of the disease.

An attempt was, therefore, made to calculate the daily intake of fluorides by the population, on the basis of water consumed, for both cooking and drinking purposes. It was a common feature of this area that water used by the inhabitants for cooking was invariably obtained from their domestic wells containing brackish water, while the drinking supplies were brought from a common source in each area. All the wells were examined for their fluoride content. Assuming that about six pints of water per head were being consumed per day, the average intake of fluoride per head in the different areas would be as follows :—

Mild area	..	Bestapalam	5·4	mg. per head per day.
Moderate area	..	Podili town	9·4	,, ,, ,,
Severe area	..	1. Baptistpalayam	13·2	,, ,, ,,
		2. Lakkisettipalayam	16·8	,, ,, ,,
		3. Malapalli	19·2	,, ,, ,,
		4. L. F. Choultry well (used regularly by a few).	24·0	,, ,, ,,

These figures point to the existence of a definite relation between the quantity of fluorides ingested and the extent and severity of intoxication observed in the area.

During these surveys the effects, if any, of occupation on the incidence and severity of the disease were also studied. Unlike the hamlets of Podili, it was possible to distribute the population of Podili town proper amongst different occupations. From the data classified on this basis, it was found that men engaged in pursuits involving light manual labour (clerks, teachers, merchants, artisans, etc.) showed a lower incidence of bone lesions and severe manifestations were found in only 9 per cent of those examined (15 out of 179) as compared with agricultural and other labourers doing hard manual work who showed a 16·2 per cent incidence of severe affections (25 out of 154).

As has already been pointed out (Shortt *et al.*, 1937*b*), the severe types of chronic fluorine intoxication were characterized by progressive changes in the osseous system. Apart from the generalized manifestation of rigidity of the spine, ribs and thoracic walls noticed in these cases, the calcification and irregular deposition of bone was observed to start at points particularly exposed in various occupations to considerable stress and strain. Thus, in basket weavers, the left wrist and arm, used largely in weaving, were the first to become affected. A similar selective action was demonstrable in the case of cobblers and other artisans and agricultural labourers. Occupation thus appeared to influence the sites of affection to some extent.

Apart from occupations, the social habits and conditions of life of the people would also appear to influence the condition. As Dean (*loc. cit.*) and Gaud and Charnot (1938) have pointed out, the relationship of fluorine intoxication to the ingestion of fluoride is dependent upon the following factors :—

1. The meteorological factors, e.g. high temperature.
2. The consumption of dirty waters laden with suspended impurities.
3. The culinary and dietetic habits of the people.

All these factors were found to operate adversely in the areas surveyed. The hot-weather conditions which normally prevail in this area for the greater part of the year not only increase the water intake but also necessitate, when the water level in wells is greatly reduced, the use of water containing abnormal amounts of sediment. The common grinding-stone made out of local rock and used universally for the preparation of foods and condiments also adds its quota of both soluble and insoluble salts. Specimens of rocks obtained from the area have been proved to contain large amounts of fluorite and fluorapatite. The rôle of these inorganic compounds in fluorine intoxication has yet to be finally determined.

Nutritional survey of villages in Kanigiri and other hamlets.

During the routine survey, it was observed that the residents of Dirisavancha village (with water sources showing a fluoride content of 2 to 2·8 parts per million) were almost entirely free from affections of bone and spine, while its *Madiga* hamlet, situated within 300 yards of the main village (with a fluoride content of about

3 parts per million in the water-supply), showed an abnormally high incidence of the affection. Kammavarpalli, another hamlet situated about a mile away from the main village (fluoride content 3 parts per million), showed a comparatively lower incidence.

The social and economic status of the inhabitants of the main village and its two hamlets differed widely. There was, therefore, reason to believe that there would be a corresponding disparity in the state of their nutrition which probably accounted for the variations noticed in the intensity of the affection in these three areas.

A detailed study was, therefore, made of the social and economic conditions and of the nutritional status of the residents of the main village and its *Madiga* hamlet.

Dirisavancha main village.

Topography.—Dirisavancha is a village with a population of about seven hundred, about eight miles away from Kanigiri town, and reached by a cart track two miles away from the main Podili-Kanigiri road, at the sixth mile from Kanigiri. The village is above the confluence of two hill streams, the Makkeru and Chinnavagu, which skirt the village on the north and south respectively, and join together a short distance beyond the village limits.

Water-supply.—The majority of the people use spring pits (*Chelamas*) dug out in the bed of the streams, Makkeru and Chinnavagu. Running river water is never used. The fluoride content of water derived from the spring pits, which are only shallow scoopings in the bed of the river not over a foot deep, varies from 2 to 2·8 parts per million. This supply is supplemented by water from step-wells and draw-wells (fluoride 2·8 parts per million) in the village which is generally used *only* for cooking purposes. Some families obtain all their water (both for cooking and drinking) from a few selected wells which have a fluoride content of 2·8 parts per million. A few families, probably not more than four or five, however, use only the spring-water from the streams both for cooking and drinking.

Social and economic conditions.—The main village of Dirisavancha is a prosperous one as judged by local standards. A number of Komatis (traders) live in this village. These are mostly dealers in cloth woven locally or obtained from weaving centres in adjoining districts. One or two adult male members of each family spend three to six months every year outside the district hawking cloth in far off places such as Bhadrachalam, etc. They follow this profession either as proprietors or as hired servants receiving monthly wages in cash from other local traders. The remaining population of this village consists of weavers, dhobies, shepherds and agriculturists. The weavers earn on an average a minimum of four annas per day. The dhobies wash clothes, each for about ten families, for which they get wages in the form of prepared food that would ordinarily suffice for their family for the noon meal. They are, in addition, the butchers of the village for slaughter of sheep ; they also help the raiyats in dressing fowl, for which services they invariably get meat in return. The Makkeru stream provides small fish for about six months in the year.

The dwelling houses of most of the inhabitants in this village comprise more than one room each and the majority have two, a living room and a kitchen. The living room in the case of a weaver's house has provision for a single hand-loom with all its appurtenances.

There are two elementary schools catering for the educational needs of the children. Although the village presents a fairly tidy appearance, the general sanitation must be deemed to be poor.

Nutrition.—A careful preliminary inquiry was made in the main village to obtain information regarding the number of individuals who complained of pain and stiffness of joints. Only five individuals, all of about 50 years of age, showed symptoms of what might be regarded as a mild fluorine intoxication. The dietaries of these families and of other representative groups—based primarily on the sources of water-supply used by them—were studied in detail.

Thus, the daily dietaries of thirteen families in Dirisavancha village representing the above-mentioned groups were assessed. The technique described by Aykroyd (1937, 1938) was followed. The various items of foodstuffs consumed daily by the family were weighed in grocery scales; the 'consumer members' of the family were carefully listed with regard to age and sex. In order to determine the 'adult value' of the family, the scale of co-efficients and 'energy requirement units' given by Aykroyd (*loc. cit.*) were adopted. The various nutrients in the daily ration were then worked out from tables furnished by Aykroyd and the quantities of various nutrients and the calorific value of the intake per consumption unit—'requirement units'—were computed. Where certain articles of food were consumed only occasionally, their average daily consumption was worked out and included in the values for daily food intake.

It is surprising to note that in this, as in most other villages of this district, milk, as such, is not consumed even by the well-to-do who maintain a large herd of milch-cattle. Similarly, eggs are not eaten at all, even by families who rear poultry in large numbers. The most surprising fact recorded by us is that, even though many of the well-to-do people owned a large number of cows, they never milked them—the calves being allowed the benefit of all the milk. The idea underlying this practice would appear to be that, by so doing, draught bulls of a sturdier and more robust type could be bred for agricultural operations. Shepherds own large flocks of sheep and goats but do not use their milk. Thus, only she-buffaloes are maintained for the supply of milk and milk products. The milk is boiled, curdled and then churned for butter and buttermilk. Only a small quantity of ghee made out of the butter so prepared is utilized in the dietary, the bulk of it being sold to traders from other parts of the district.

A few important considerations will have to be borne in mind in assessing the results of this and other surveys included in this paper :—

(*a*) *Incidence rate.*—Particular care was taken to see that the incidence rate for chronic intoxication as affecting bones was based on random sampling of families and calculated with reference *only* to persons exposed to risk, i.e. persons of the ages of 21 to 75.

(*b*) *Nutritional survey.*—The rôle of carotene (vitamin A factor) is perhaps also a contributing factor, but being closely associated with *vitamin C* in leafy vegetables, it stands to rise or fall along with the latter in the dietaries of the families studied. There are, however, a number of limitations that have to be taken into account in any correct appreciation of the availability and nutritional value of some of these factors. The following points have, therefore, to be kept in mind in the assay :—

(*i*) The evaluation of the dietary from day to day for a week was not made in this survey as no variation occurred in the diet of these people. It was a monotonous diet, as most of the villages and hamlets had no access to bazaars and had to rely entirely on the itinerant pedlar for their minor requirements. The transactions were entirely by a system of barter.

(*ii*) The only sources of vitamin C in the large majority of cases in all the villages surveyed, are dried chillies and leafy vegetables such as Thotakura (*Amaranthus gangeticus*), Gonkura (*Hibiscus sabdariffa*), etc. Though red chillies are consumed almost universally with every meal, the leafy vegetables are taken once, twice or thrice a week by people according to their means and even that for only two to three months in the year when these vegetables are available. The inclusion in dietary values of this item by distributing the two months' consumption over the twelve, and the evaluation of the vitamin C intake on that basis, are obviously very fallacious for the reason that these people do not get any vitamin C from this source continuously for ten months in the year. The red chillies (*Capsicum annuum*) thus constitute the only source of their vitamin C intake all the year round which is, therefore, very little. Continued vitamin C deficiency is, therefore, the rule in all these cases, particularly with reference to the very poor classes.

(*iii*) Further, vitamin C being liable to rapid destruction by boiling and most green leafy vegetables being usually eaten only after prolonged heating and boiling—in many cases even the organic salts are rejected with the boiled water—the loss of vitamin C content in this process would necessarily be high and cannot be properly assessed. Allowance for such losses have not been made in our estimates and calculations of dietary values. Consequently, the C values in our statements represent the maximum possible but not the actual intake available to the body. The only green leaf consumed raw is the betel leaf (*Piper betel*). Even this was a poor half-dried or crinkled specimen and did not materially add to the vitamin C intake ; the itinerant pedlar buys his wares once a week in the cheapest market and sells them to the villagers during a whole week.

(*iv*) Thotakura—(*Amaranthus gangeticus*)—is reported to contribute 2,500 to 11,000 units of carotene. In the absence of any definite information as to what value is to be chosen in this very wide range for any local variety, we have taken the higher figure of 11,000 in our calculations. This again adds another fallacy.

A few representative dietaries of the different groups of families included in the survey are furnished in the *Appendix*. The calculated dietetic values of the diets from thirteen families are given in Table I :—

Table I.

Calculated dietetic values of diets of families in :—

Dirisavancha—main Hindu village.

(Allowing for leafy vegetables, etc. consumed for about two to four months in a year.)

Number.	Family.	Number of adults.	Economic and financial condition.	Proteins.	Fat.	Carbohydrates.	Calcium.	Phosphorus.	Iron.	Calories.	Carotene.	Vitamins A	Vitamins B_1	Vitamins B_2	Vitamin C.
1	Weaver ..	4	Fair	97·8	34·5	664·6	1·9	3·27	77·5	3178·6	4641·0	2·3	565·0	++	87·0
2	Agriculturist ..	34	Rich	161·5	50·0	1001·0	2·58	4·39	110·7	5089·8	4709·6	2·3	615·0	++	88·0
3	Komati (Vysia) ..	6	Fair	92·2	39·7	420·3	0·71	2·30	60·2	3431·2	4641·0	..	918·3	++	75·3
4	do. ..	12	Poor	82·4	19·9	562·4	1·78	2·73	70·4	2939·6	3942·0	..	442·8	++	74·2
5	Weaver ..	3	Fair	111·6	44·6	763·2	2·20	3·64	88·4	3926·2	4827·0	..	663·8	++	75·6
6	Dhobie cum butcher	5	,,	100·6	43·2	565·3	1·72	2·85	67·8	3049·8	4493·8	2·8	558·3	++	75·0
7	Komati (Vysia) ..	6	Poor	74·2	28·1	498·6	1·52	1·48	56·7	2549·3	3040·0	..	402·0	+	46·5
8	do. ..	10	Rich (rice eaters).	89·9	52·9	581·9	1·17	1·65	42·6	3234·2	6786·0	5·2	775·0	++	186·0
9	do. ..	8	Rich but a miser	85·4	26·4	579·6	0·87	1·64	46·2	2962·3	6375·0	3·0	746·0	++	113·5
10	Brahmin priest ..	4	Poor	59·5	37·8	394·2	0·53	1·18	27·1	2150·8	3910·0	2·8	484·0	++	75·0
11	Dhobie cum butcher	15	Fair	73·6	41·4	562·6	1·78	2·75	56·4	2844·0	4380·0	2·3	529·6	+	46·5
12	Weaver ..	4	,,	104·2	34·7	718·4	1·92	3·19	76·5	3598·0	3538·0	2·3	595·7	+	46·0
13	Government servant	5	Well-to-do	113·5	42·5	629·2	1·06	2·48	60·4	3331·2	4896·0	4·4	958·3	++	75·0
	Average ..			95·9	38·1	610·9	1·5	2·6	64·7	3252·7	4628·8	2·1	634·9		89·7

From a study of the dietary records, it will be seen that ragi (*Eleusine coracana*) and sajja (cumbhu) (*Pennisetum typhoideum*) constituted the staple articles of food—only a few Brahmin and Komatis (Vysias) using home-pounded rice (*Oryza sativa*). Out of thirteen families comprised in this study, all except one got between 2,500 and 3,500 calories per 'requirement unit'. The protein consumed ranged from 59·5 g. to 161·5 g. per unit per day and was almost entirely of vegetable origin in the majority of instances. In the case of non-vegetarian families, however, buttermilk, meat, fish and very occasionally pork, contributed a small fraction of the protein requirement. Taking the average energy requirement to be 2,500 calories for the population under study, twelve out of the thirteen families were found to get their full energy requirements. These were, however, derived largely from carbohydrates—fats contributing very little and even this little being made up of fats derived chiefly from sajja or ragi.

ADEQUACY OR OTHERWISE OF NUTRIENTS : MINERALS, CALCIUM AND PHOSPHORUS.

These are the chief 'inorganic substances entering into the composition of the skeletal structure of the body'. Taking as normal requirements, 0·68 g. of calcium and 1 g. of phosphorus per unit per day, the diets studied by us were found to furnish from 0·5 g. to 2·5 g. per unit—only one family consuming less than the average daily requirement of 0·68 g.—of calcium. The phosphorus requirements were fully satisfied in the case of all the families.

It must be noted, however, that the major portion of the calcium was derived from staple food grains (ragi and sajja) and from vegetables and only very little from milk and milk products; this factor is important in assessing the assimilability of the calcium provided since it is believed that calcium derived from vegetable sources is not as readily assimilated as that from milk and milk products.

Iron.—The diets of the families studied, were found to provide iron ranging from 27 g. to 110 g. as against the standard of 20 mg. required per unit per day.

Vitamins.—Very little of vitamin A derived from meat and from milk and milk products was available for the families. The carotene content, however, of their dietaries appeared to be normal. The intake of vitamins B_1 and B_2 was more or less adequate. The vitamin C requirement was derived chiefly from dried chillies and in some cases from leafy vegetables. While only one family obtained its full requirement, the rest were below normal. A detailed reference to this factor will be made later. The majority of the people did not get any vitamin D from the dietary.

It will thus be seen that the diet of the representative families studied in Dirisavancha, though adequate as far as energy requirements are concerned, was still not well balanced.

MADIGAPALLI HAMLET OF DIRISAVANCHA.

A similar survey was carried out in Madigapalli hamlet where the incidence and severity of fluorine intoxication was high. This hamlet, situated about five hundred yards away from the main village, has twenty-two huts with a population of about one hundred.

The huts are fairly evenly distributed, with pathways all round leading from one cluster to another. The sanitation of the hamlet is similar to that described under Dirisavancha above. The source of water-supply at present is a step-well on the bank of the Chinnavagu, about three to four hundred yards away from the hamlet. This well has been in use for the last twenty-five years and has a fluoride content of 3·2 parts per million. Another step-well which, in its present state of disuse for over twenty-five years, shows a fluoride content of 3·5 parts per million, was the main source of supply to this hamlet before the present one was brought into use. The residents of this hamlet are all *Madigas*. Their personal hygiene is of a very low standard as compared with that of the inhabitants of the main village. They are poorly clothed, ill-nourished and the majority of them look famished. They do not obtain their water from the spring pits in the streams either for cooking or for drinking. With the exception of three individuals who are employed, two as school masters and one as a village servant, the rest are all agricultural labourers and, in addition, ply the cobbler's trade. Their economic condition is very low indeed.

The majority of the people are on a semi-starvation diet, only one in each of many families being an earning member. When work is not available, he has either to borrow or to starve.

The average income of most of the families in this hamlet ranges in terms of money value (from Re. 0–0–6 to Re. 0–2–6 per day *per family*; exceptions to this are the village servant and the two school masters who are the only persons in the hamlet in receipt of monthly wages in cash).

Only a few of the residents own buffaloes—never more than one per family. A few rear poultry but no sheep or goats.

Eggs are not consumed by any of them and carrion also is not eaten. Meat is obtained very occasionally on a co-operative basis—probably not oftener than once a month—and distributed amongst all the residents of the hamlet.

Two pieces of cloth for the adult male and one cheap *saree* for each of the adult females constitute the entire wearing apparel for a whole year. Children up to 7 or 8 years of age are very scantily clothed. The staple article of diet is ragi. Sajja being more expensive is rarely, if ever, eaten. The only other constant articles in their diet are chillies of inferior quality and salt, which are obtained by a system of barter from a pedlar from the main village.

Nutrition.—A specimen of the dietary used by the ten families studied is given in the *Appendix*. The calculated dietetic values for the ten families are given in Table II. It will be seen that only four of the ten representative families were getting more than the average energy requirements—2,500 calories per unit per day—the remaining families securing only between 1,047 and 2,200 calories.

TABLE II.

Calculated dietetic values of diets of families in:—

Madigapalli of Dirisavancha.

Number.	Number of adults.	Economic and financial condition.	Proteins.	Fat.	Carbohydrates.	Calcium.	Phosphorus.	Iron.	Calories.	Carotene.	Vitamins A	Vitamins B_1	Vitamins B_2	Vitamin C.
1	7	Well off	77·3	25·6	590·8	1·84	2·85	61·2	2898·7	3664·6	Practically *nil*.	402·0	Poor	40·2
2	2	„	98·0	31·2	660·2	1·75	2·94	74·4	3297·1	3977·1		596·0	„	50·0
3	6	Very poor	30·0	5·8	308·3	1·52	1·11	25·7	1412·6	337·6		39·1	„	5·2
4	7	„	22·1	4·2	229·5	1·20	0·82	16·0	1047·3	229·3		22·0	„	2·5
5	5	Well off	91·0	31·6	676·4	1·67	3·30	67·8	3168·2	3377·6		87·5	„	50·0
6	8	Medium	50·5	9·4	533·7	2·11	1·82	38·0	2439·1	519·0		22·0	„	5·2
7	2	Very well off	90·0	32·4	623·8	1·83	3·18	74·0	3127·6	4364·0		506·0	„	51·8
8	4	Very poor	36·3	6·8	381·6	1·60	1·30	26·6	1738·0	379·0		11·3	„	2·6
9	6	Ordinary labourer	44·2	8·4	459·1	1·87	1·63	32·0	2094·6	477·6		16·0	„	2·6
10	7	Very poor	32·8	6·1	342·6	1·54	1·22	23·9	1567·0	344·0		9·0	„	2·6
AVERAGE	..		57·2	16·1	480·6	1·69	2·02	43·96	2279·02	1767·0		170·5		21·3

Even this calorific value of the diet was furnished mostly by carbohydrate, the proteins which were mainly of vegetable origin, contributing less than the minimum requirements. As against a minimum of 59·5 g. per unit of protein (mainly of vegetable origin) consumed by the Dirisavancha residents, the *Madigas* secured only a minimum of 22 g. per unit per day. Even with these poor diets, it should be noted that calcium, phosphorus and iron were taken daily in quantities which would be considered satisfactory.

Only three families were able to obtain between 402 and 596 International Units of vitamin B_1; the rest did not get sufficient vitamin B_1 in their diet.

The supply of vitamin C was very deficient, ranging from 2·6 mg. to 51·8 mg. (average 21·3) compared with an average of 89·7 mg. in the case of the Dirisavancha residents. Animal protein was almost entirely absent from their diets. Dry chillies alone furnished the little vitamin C they obtained, as their poverty prevented the purchase and use of vegetables, even when available. Even tamarind fruit was a luxury with them; only a few could afford it once or twice a week, while the rest were obliged to resort to tender tamarind leaves, whenever available, to serve as a poor substitute for the fruit.

A survey of the nutritional status of the children with particular reference to vitamin deficiencies.

A survey of the nutritional status of children in Dirisavancha main village and its Madigapalli hamlet revealed the following features :—

1. Phrynoderma (vitamin A deficiency) was noticed to a much greater extent in the *Madiga* children (60 per cent) than in the Hindu children of the Dirisavancha main village (27·6 per cent).
2. Xerosis (vitamin B deficiency) was universally present in varying degrees of severity, in all the children of both groups examined, as detailed below :—

	Total.	Severe.	Moderate.	Mild.	Very mild.
Dirisavancha ..	21	4	3	12	2
Madigapalli ...	20	1	7	10	2

3. Angular stomatitis (vitamin B_2 deficiency) was more prevalent amongst the *Madiga* children (90 per cent) than in the other group (57·1 per cent).
4. Gingivitis (vitamin C deficiency) was almost the same in both groups (70 per cent).

5. Rickets (vitamin D deficiency) was noticeable in about 70 per cent of the children in both groups but the severity was more marked in the *Madiga* group.

6. Forty per cent of the *Madiga* children and 19 per cent of the Dirisavancha children were found to be anæmic.

7. The height to weight ratios showed higher values for the Dirisavancha group than for the *Madiga* group.

8. All the children of the ages of 5 to 14 in both groups showed mottling of the enamel of the permanent teeth. It is interesting to note that nearly 40 per cent of the Dirisavancha children and only 25 per cent of the *Madiga* group showed the severe type of mottling. The incidence of the mild type of mottling in the Dirisavancha children was nearly twice as much as in the *Madiga* children. Deciduous teeth also showed mottling in all the children of both groups.

While the nutritional status of the children was definitely *below par* in both the groups, the children of the *Madigas* were definitely undernourished and showed a greater all round deficiency than the children of the other group.

Discussion.

(a) Comparison of Dirisavancha main village with its Madigapalli hamlet.

The incidence of chronic fluorine intoxication as evidenced by bone lesions is high in Madigapalli where the drinking and cooking water is derived from a single step-well with a fluoride content of 3·2 parts per million, while the incidence in the Dirisavancha main village (where the water-supplies from different sources show an average fluoride content of 2·5 to 2·8 parts per million) is very slight and the few cases (five out of seven hundred) show only a very mild affection. The difference in the incidence and severity between these two groups is striking in spite of the fact that the *Madigas* have only a slightly higher fluoride (0·4 part per million) intake from their water. In this connection, it has to be noted that the inhabitants of Dirisavancha (the higher castes) ordinarily cook three to four dishes per day with water containing 2·8 parts per million of fluoride, take a comparatively larger quantity of food and salt with it and, therefore, probably also drink more water both as water and with their buttermilk. Again if, as is probable, the foodstuffs grown in the area also contain fluoride in appreciable amounts, the fluoride intake by the Dirisavancha residents would be still higher. Consequently, they are likely to ingest a quantity of fluorides which would approximate to that of the *Madigas*, or would perhaps be even slightly higher. The comparatively high incidence of the mild and severe types of mottling of the teeth in the Dirisavancha children would also appear to lend support to the view that the fluoride intake of this group is perhaps higher than that of the *Madigas*. The differences in nutrition recorded above in detail, would thus appear to be the only other factor that

could account for the difference in the incidence of chronic fluorine intoxication between the two large groups consuming nearly the same fluoride content in water. From the data available, it should be observed that even in the case of the *Madigas*, the calcium intake—not calcium derived from milk or vegetables but exclusively from ragi—is well above the usual textbook figures for calcium requirements, viz. 0·68 g. daily for adults and 1 g. for children. The calcium intake of the Hindu population of Dirisavancha does not differ very much from that of the *Madigas*, the only difference being that the former get their calcium requirements to some extent from buttermilk and vegetables, if not daily, at occasional intervals. When the dietetic tables of the two groups are compared, it will be seen that while the vitamin C intake for the Hindu population of Dirisavancha is about 89·7, that of the *Madigas* averages only about 21·3. When it is remembered that a well-balanced diet for children and adults should contain about 170 mg. of vitamin C per day, the serious deficiency in the intake of this factor by the *Madigas* is readily apparent.

During the progress of this survey, we found that in the *Madiga* hamlet of Kanigiri town (with a fluoride content of only 2 parts per million in their water-supply) bone affections were fairly prevalent. Indeed from the estimation of fluoride in waters in the Nellore district and from a study of the incidence of cases in several villages, it was noted that villages where the water-supplies showed a fluoride content of 2 to 3 parts per million consistently showed an incidence of chronic fluorine intoxication. It would, therefore, appear that, as revealed by the nutritional survey of the Dirisavancha village and its hamlet Madigapalli, a nutritional deficiency (particularly of vitamin C) played an important rôle as a contributory factor, in chronic fluorine intoxication. The nutritional surveys were, therefore, extended to other representative villages for purposes of comparison with particular reference to the influence of the vitamin C factor.

The following villages were thus surveyed :—

1. Kammavarpalli of Dirisavancha.
2. Madigapalli of Kanigiri.
3. Parvathipuram of Podili.
4. Baptistpalayam of Podili.
5. Lakkisettipalayam of Podili.
6. Mittatmakur of Gudur.

Particulars of the detailed nutritional surveys are not, however, given here. Table III gives the nutritional index of the families in each of the villages based on the averages for each constituent of the food intake. The fluoride content of the water-supplies and the percentage incidence of chronic fluorine intoxication in each of the villages are also included in this table.

The results of the surveys in these villages will be compared one with another with particular reference to the fluoride content of waters and the vitamin C intakes of the residents in the respective villages.

TABLE III.

Nutritional index of families in each of the villages surveyed.

Averages for the eight hamlets surveyed.

Number.	Name of hamlet and village.	Fluoride content of drinking water (p.p.m.).	Percentage incidence of chronic fluorosis.	Proteins.	Fat.	Carbohydrates.	Calcium.	Phosphorus.	Iron.	Calories.	Carotene.	Vitamins A	Vitamins B_1	Vitamins B_2	Vitamin C.
1	Dirisavancha (main) (Hindus).	2·4	4·4	95·9	38·1	610·9	1·5	2·6	64·7	3252·7	4628·8	2·1	634·9	++	89·7
2	Mittatmakur—Gudur (Madigavada).	2·2	13·0	96·7	18·3	672·8	0·52	2·85	64·7	3226·7	5239·9	5·7	15·9	++	70·6
3	Kammavarpalli—Dirisavancha (Hindus).	3·1	44·5	88·4	28·5	712·6	2·22	2·9	68·2	3471·0	2225·0	0·4	368·1	++	29·7
4	Madigapalli—Dirisavancha (Madigas).	3·0	60·0	57·2	16·1	480·6	1·7	2·0	43·9	2279·0	1767·0	..	170·5	+	21·3
5	Madigapalli—Kanigiri (Madigas).	1·6	40·4	62·7	15·2	542·8	1·93	2·0	42·9	2538·6	1844·2	24·0	122·4	+	26·2
6	Parvathipuram—Podili (Hindus).	2·4	60·3	90·7	24·2	747·3	2·2	2·9	64·2	3558·4	1681·3	1·6	429·5	++	18·9
7	Baptistpalayam—Podili (Christian Madigas).	2·8	78·6	58·4	11·0	616·4	2·7	2·2	44·7	2799·4	1067·3	0·5	2·5	+	11·1
8	Lakkisettipalayam—Podili (Jangams).	2·2	60·0	60·4	11·3	634·0	2·66	2·3	45·4	2879·3	748·6	0·3	2·1	+	7·2
	Requirements for a well-balanced diet.			73·0	74·0	408·0	1·02	1·47	44·0	2590·0	7000·0		400·0		170·0

(b) Comparison of Dirisavancha main village with the Madigapalli hamlet of Kanigiri.

This comparison is of considerable significance. Madigapalli of Kanigiri has a single source of water-supply (the Magani well) with a fluoride content varying seasonally from 1·4 to 2·2 parts per million (average 1·6 parts per million, i.e. about 33⅓ per cent lower than that of Dirisavancha main village). Still, the incidence of bone trouble in this hamlet (40·4 per cent) is very much higher than that in Dirisavancha with 4·4 per cent. This higher incidence is found to co-exist with a lower nutritional and economic status of the residents of Madigapalli, as compared with that of the Dirisavancha inhabitants. The average calcium intake for Dirisavancha is 1·5 as compared with 1·93 for Madigapalli of Kanigiri. The average vitamin C value for Madigapalli of Kanigiri is low, being only 26·2 even though no deduction has been made for loss in cooking and the maximum quantities and values for *amaranth* have been allowed in calculation.

(c) Comparison of Madigapalli of Kanigiri with the Madigapalli of Dirisavancha.

There are no bed-ridden cases in Madigapalli of Kanigiri and the general incidence rates are 44·4 and 60·0 per cent respectively for the two hamlets. The vitamin C intake is almost the same for both. The lower incidence in the Madigapalli of Kanigiri is manifestly due to the following factors, viz. :—

(*i*) The diets show a higher calorific value.

(*ii*) The *Madigas* of Kanigiri receive better all-round nourishment.

(*iii*) Starvation is very much less amongst these people as they live in a quasi-urban area with better prospects for continuous labour from day to day.

(*iv*) They earn wages in cash which permits of purchase of their requirements with greater facility and ease and more cheaply. Food is available more readily and in greater variety in Kanigiri when obtained for cash.

(*v*) The fluoride content of the Magani (Kanigiri) well is about one half of that of the step-well used by the *Madigas* of Dirisavancha.

(d) Comparison of Kammavarpalli of Dirisavancha with the Madigapalli hamlet of Dirisavancha.

The fluoride contents of the water-supplies used for domestic purposes are almost identical, being in the neighbourhood of 3 parts per million. This has probably exceeded 3 parts per million in Kammavarpalli during recent years as the new well with a fluoride content of 5 parts per million and over has had to be universally used for cooking and often also for drinking when the spring pits in the river bed dried up. The incidence of bone troubles for Kammavarpalli is 44·5 per cent as against 60 per cent for Madigapalli of Dirisavancha.

The incidence rate according to age-groups shows none out of twenty-six in Kammavarpalli as against six out of thirty-eight in Madigapalli in the 21 to 30 group. The 31 to 40 group shows an almost equal incidence ; in the 41 to 50 group, Kammavarpalli shows six out of twenty-six as against fourteen out of thirty-eight in Madigapalli. A larger number of people are found to be affected in the 51 to 60 and 61 to 70 age-groups in Kammavarpalli than in Madigapalli of Dirisavancha. This is probably due to a survival of a large number of the affected people as a result of better nutrition of the Kammavarpalli residents. The severity of the infection is definitely lower in Kammavarpalli than in Madigapalli as will be seen from the following table :—

TABLE IV.

Name of the village.	Total number of cases.	Mild.	Moderate	Severe.	Bed-ridden.
Kammavarpalli ..	26	24	*Nil*	1	1
Madigapalli ..	38	22	5	6	5

Twenty-four of the twenty-six cases in Kammavarpalli are really very mild showing only pain on pressure in the lumbo-sacral region and in some cases also in the neighbourhood of the ankle joints. In a great many of these cases, the symptoms are only of about two years' duration—probably due, as already stated, to the higher intake of fluorides from the new well-water. The existence of a definite relationship between a high fluoride and low vitamin C intake in the production of bone troubles is thus made apparent.

In the survey of Mittatmakur (a *Madiga* hamlet in Gudur taluk), which served as a control, to the other villages discussed above, the following data were collected :—

The average fluoride content of the water of the well used by the residents was 2·2 parts per million.

A dental survey of the children of the ages 5 to 14 revealed the presence of mottling of the enamel of the teeth in varying degrees of severity.

The general incidence of bone cases was very low. Of twenty-four adults examined, five were found to show bone affection ; these were all between 51 and 60 years of age. Three of them were very mild and two moderate with only slight limitations of the movement of the neck and other joints. There was not a single advanced or bed-ridden case in this hamlet.

The economic status of these *Madiga* families in Mittatmakur is very much better than that of similar groups of families in the northern taluks of the district ; the monthly cash earnings of the former ranged from Rs. 4 to Rs. 17-8. According to local standards applicable to this class of people, a family would be deemed to be poor if the monthly income was less than Rs. 7-8, i.e. Re. 0-4-0 per day ;

of moderate means, if the income was from Rs. 7-8 to Rs. 15 and well-to-do if over Rs. 15 per month. On the above basis, three of the families were classed as poor, two as well-to-do and the rest as of moderate means. The higher economic status prevailing in this hamlet was manifestly due to the following factors :—

1. Certainty of labour for the men-folk in mica mines and paddy fields, the lowest adult earning being Re. 0-2-0 per day.
2. The existence in the village of cottage industries for women (e.g. mica splitting, weeding in paddy fields, etc.) ensuring an average daily cash income of at least Re. 0-1-0 per day.
3. A regular supply of one and occasionally two meals by the proprietor to the field labourers, while engaged in cultivation operations.
4. Poultry-farming; sale of eggs and fowls by some families adding to the family income.
5. Fresh fish is available for six months in the year and used by the people.
6. Leafy vegetables are available for about four to six months in the year and are used in larger quantities.

The absence of severe manifestation of fluorine intoxication in this area is, therefore, to be explained by the higher nutritional level of the dietaries, including a higher intake of vitamin C as compared with similar classes of people in the other hamlets.

Salient features brought out by the survey.

From a careful study of the data collected during the surveys, the following observations appear to be of special significance :—

1. Even with a high fluoride content of the water used for drinking and cooking, the incidence and severity of chronic fluorine intoxication was greatly influenced for the better, by an intake of nutrients which approximated to accepted International standards of sufficiency.

2. Even with a comparatively lower fluoride content in the water used for domestic purposes, a deficiency in the intake of nutrients resulted in a higher and more severe incidence of the disease, the younger adult age-groups also becoming affected (*cf.* Kammavarpalli and Madigapalli of Dirisavancha).

3. Where the fluoride content of the water was approximately the same in two or more villages, the incidence of the affection was influenced to a large extent by the intake of vitamin C in the diet, i.e. the higher the intake, the lower the incidence and vice versa (*cf.* Mittatmakur and Dirisavancha with the other villages).

4. Ragi was the staple article of diet with the poor *Madigas*, while ragi and sajja (bajra or cumbhu) were consumed by the middle and upper middle classes in Podili and Kanigiri taluks. Rice was eaten very occasionally during

feasts and festivals only. Cholam (*Sorghum vulgare*) was the staple food for the *Madigas* of Mittatmakur in Gudur taluk and, as already stated, they were able to obtain a fairly good supply of a variety of greens for four to six months in the year. The calcium content of the staple grains are as under :—

Ragi	.. 0·33 per cent.	Rice	.. 0·01 per cent.
Sajja	.. 0·05 per cent.	Cholam	.. 0·03 per cent.

Ragi is seven times as rich in calcium as sajja (bajra or cumbhu), eleven times as rich as cholam and thirty-three times as rich as rice. The *Madigas* and the poor caste Hindus who had necessarily to live on ragi, the cheapest of the four staple grains, therefore, obtained in their diet a very much higher quantity of calcium than is necessary.

The part played by calcium in the absence of an adequate supply of vitamin D in the diet would, therefore, require further investigation.

Summary and conclusions.

A detailed study of the factors involved in the production of 'mottled enamel' in children and severe bone manifestations in adults, as a result of the ingestion of fluorides in drinking water, has been made during the last three years. This study revealed :—

1. 'Mottled enamel' in children was universally present in all the areas where the fluoride content of the water was in the neighbourhood of or exceeded 1 part per million. The severity of the condition was in proportion to the fluoride content.

2. Deciduous teeth were also affected in areas with a high fluoride content and severe type of mottling.

3. The severe form of chronic fluorine intoxication as affecting bones, ligaments and joints was restricted to some villages situated in the three northern taluks of the Nellore district.

4. This area, which contained fluorite and fluorapatite as constituents of the rocks, showed the highest fluoride content (6 parts per million) in its water-supplies.

5. A continued residence of over fifteen years in the endemic area was necessary in the case of *adults* to bring about symptoms of chronic intoxication.

6. The severity and site of the lesions depended to some extent on occupation.

7. A disparity in the incidence was noted in certain villages in which the fluoride content of the water was more or less identical.

8. Economic and nutritional surveys were carried out in several representative villages. The incidence and severity of the disease had a definite relation to the economic and nutritional status of the communities.

9. A pronounced deficiency of the vitamin C factor in the diet was especially associated with a severe incidence of the disease.

10. The two important factors concerned in the production of severe chronic fluorine intoxication in human beings in this district were the high fluoride content of the domestic water-supplies and a pronounced C avitaminosis.

ACKNOWLEDGMENTS.

We are indebted to Lieut.-Colonel C. M. Ganapathy, M.C., D.P.H., I.M.S., Director of Public Health, Madras, for his valuable suggestions in the conduct of the surveys. Our thanks are also due to Dr. R. Adiseshan, Assistant Director of Public Health, for his suggestions in connection with the economic and nutritional survey of some of the villages. The work was greatly facilitated by the uniform and willing co-operation of the public health staff of the various districts.

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APPENDIX.

Illustrative dietaries of the inhabitants of Dirisavancha and Madigapalli.

I. *Dirisavancha :—*

(*a*) *Daily dietary of a well-to-do joint family of* 34 *members* (13 *adults,* 17 *children and* 4 *servants*).

Total income	about Rs. 250 p.m.
Daily staple diet :—	
Ragi (*Eleusine coracana*)	27 lb.
Sajja (*Pennisetum typhoideum*) or cholam (*Sorghum vulgare*) ..	48 lb.
Chillies (dry) (*Capsicum annuum*)	6 lb.
Tamarind (*Tamarindus indicus*)	12 oz.
Dhal (*Cajanus indicus*)	7 lb.
Salt	As required.
Ghee	3 oz.
Buttermilk (thin)	40 pints.

Vegetables when available for 2 to 3 months in a year.

Daily :	Brinjals (*Solanum melongena*), Lady's finger (*Hibiscus esculentus*) ..	6 lb. each.
	Greens: Gonkura (*Hibiscus sabdariffa*) ..	2 lb.
	Greens: Amaranth (*Amaranthus gangeticus*)	8 lb.
Weekly :	Gourd (*Benincasa cerifera*), Pumpkin (*Cucurbita maxima*) ..	8 lb.
	Green chillies (*Capsicum annuum*) ..	4 oz.
Occasionally :	Sheep's meat	30 lb. once a month.
	Fowl	12 lb. once in two months.

(b) Daily dietary of a middle-class family of 5 members (strict vegetarians, 3 adults and 2 children).

Total income	.. Rs. 15 p.m.
Daily staple diet :—	
Rice	.. 3½ lb.
Sajja	.. 4 lb.
Ghee	.. 2 oz.
Buttermilk (thin) ..	.. 5 pints.
Curds	.. ½ pint.
Dhal	.. 6 oz.
Chillies (dry) ..	.. 5 oz.
Salt	.. As required.

Vegetables when available for 2 to 3 months in a year.

Daily :	Brinjals .. Lady's finger ..	.. 6 oz. each.
Weekly :	Gourd	.. 1 lb.
Greens	Green chillies	.. 1 oz.
	Gonkura	.. 4 oz.
	Amaranth	.. 8 oz.

(c) Daily dietary of a poor Hindu family of 15 members (8 adults and 7 children).

Total income	.. Rs. 7-8 p.m. (including wages in kind).
Daily staple diet :—	
Ragi or sajja ..	.. 13 lb.
Chillies	.. 3 oz.
Salt	.. As required.

Vegetables when available for 2 to 3 months in a year.

Twice a week :	Greens .	.. 12 oz.
Once a week :	Brinjals, etc.	.. 12 oz.
Occasionally :	Tamarind ..	.. 8 oz. once a fortnight.
	Meat ..	.. 12 oz. once a week.
	Fowl ..	.. 8 oz. once a month.

II. *Madigapalli :—*

(*a*) *Daily dietary of a well-to-do* Madiga *family of* 6 *members* (4 *adults and* 2 *children*).

Total income : .. .. .. Rs. 17 p.m.

Daily staple diet :—

Ragi	10 lb.	
Sajja	10 lb.	
Dhal	3 oz.	Every other day.
Tamarind	3 oz.	
Chillies	4 oz.	
Buttermilk (thin)	4 pints.	

Vegetables when available for 2 *to* 3 *months in a year.*

Greens: Amaranth	8 oz.	Daily.
Greens: Gônkura	4 oz.	
Brinjals	8 oz.	
Beef	8 oz. once a week.	
Pork	1 lb. twice a year.	

(*b*) *Daily dietary of an ordinary poor* Madiga *family of* 8 *members* (3 *adults and* 5 *children*).

Total income : .. .. .. Rs. 4-12 p.m.

Daily staple diet :—

Ragi	6 to 12 lb. according to availability of work.
Chillies	1½ oz.
Salt	As required.

Occasionally : Tamarind	4 oz.	Once a month.
Beef	12 oz.	

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ENDEMIC FLUOROSIS IN SOUTH INDIA.

EXPERIMENTAL PRODUCTION OF CHRONIC FLUORINE INTOXICATION IN MONKEYS (*MACACA RADIATA*).

BY

C. G. PANDIT, M.B., B.S., Ph.D., D.P.H., D.T.M.,

AND

D. NARAYANA RAO, M.B., B.S.

(An Inquiry under the Indian Research Fund Association.)

(From the King Institute of Preventive Medicine, Guindy, Madras.)

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THE condition of chronic fluorine intoxication involving bones and ligaments, as observed by Roholm (1937) amongst cryolite workers in Denmark, was shown to occur under natural conditions amongst the people of a restricted area in the Nellore district (South India), due to the presence of fluorides in domestic water-supplies (Shortt *et al.*, 1937*a*, *b*). The detailed nutritional and other surveys carried out in this area showed that two factors were concerned in the production of these symptoms, viz. (*a*) high fluoride content of water-supplies (3 to 6 parts per million) and (*b*) a pronounced deficiency in the vitamin C intake in the diet of the inhabitants (Pandit *et al.*, 1940). Experiments were, therefore, initiated in monkeys (*M. radiata*) to induce similar changes by feeding them with sodium fluoride and to ascertain the rôle played, if any, by vitamin C in the diet, in modifying the pathological effects. Monkeys were chosen for the experiment since it has been stated that they are not able to synthesize vitamin C in the system and have to depend entirely on diet for their vitamin C requirements. The results obtained are recorded in this paper.

MATERIALS AND METHODS.

Observations were made on six monkeys, divided into three groups as follows :—

Group A.—Monkeys Nos. 1 and 2.

These received standard diet including vitamin C. No fluorine was given. This group served as a control to ascertain that the diet

given was adequate to maintain the animals in good health during the period of observation.

Group B.—Monkeys Nos. 3 and 4.

These received standard diet including vitamin C. Sodium fluoride was also given in the manner to be described later.

Group C.—Monkeys Nos. 5 and 6.

These received standard diet but *without* vitamin C. Sodium fluoride was given as indicated later.

Standard diet.—The composition and the mode of administration were as follows :—

A. Fifty grammes of bread in the morning for each monkey, excepting Nos. 5 and 6 which received 75 g. to make up for the deficiency in bulk and carbohydrates which the other monkeys obtained from fruits and vegetables.

		g.
B.	Wheat flour	210
	'Klim' (dried milk powder) ..	45
	Salt mixture (Osborne and Mendel. without sodium fluoride) ..	12
	Cane sugar.. . ..	12
	Cod-liver oil	6
	Yeast	9

This was made into six cakes. each monkey receiving one cake at noon.

C. Pea-nuts (unhusked) .. .. 25 g. in the afternoon

It is to be noted that sodium fluoride 0·062 g. included in the formula by Osborne and Mendel was here omitted.

Source of vitamin C.—This was not given in the form of ascorbic acid but was given in the form of raw green vegetables, as follows :—

	g.
Cabbage	40
French beans	30
Carrots	30
Banana	90

ADMINISTRATION OF SODIUM FLUORIDE.

The primary object of this investigation was to study the assimilation of sodium fluoride and its excretion. It was considered desirable, therefore, to give it by the stomach tube in order to ensure that the animal received the full intended dose.

A soft rubber catheter No. 6 was used for the purpose. A stock solution of sodium fluoride in distilled water 2 mg. in 1 c.c. was used. The required dose was then injected into the stomach by means of a syringe and the fluid finally washed down by a few cubic centimetres of distilled water. For the first five days,

a dose of 20 mg. of sodium fluoride per kg. of body-weight was given to each monkey 15 minutes after the noon meal. The animals, however, developed diarrhœa. The dose was then reduced to 10 mg. per kg. of body-weight. After four months, when the animals were becoming asthenic, the dose was further reduced to 5 mg.

Biochemical investigations.

It must be noted that owing to the considerable amount of work involved in the estimation of fluorine in urine and fæces daily, it was not possible to conduct the biochemical tests mentioned below on all the monkeys under observation. These examinations were restricted to three monkeys only, viz. Nos. 1, 3 and 5, i.e. one in each of the three groups noted. In arranging the monkeys in the three groups, their weights were primarily taken into consideration. The weights of monkeys Nos. 1, 3 and 5 were 2,540 g., 2,475 g., and 2,920 g., respectively. These had erupted their permanent incisors and canines. It could be regarded, therefore, that they were approximately of the same age. The weights of monkeys Nos. 2, 4 and 6 ranged from 2,440 g. to 2,740 g. They had deciduous teeth.

It was decided to study the excretion of fluorine in urine and fæces separately. For this purpose, a special metabolic stand was devised to hold the monkey cages in use at the Institute. The slope of the floor of the metabolic cage was so adjusted that while the urine drained off easily into the special receptacle provided, the fæces were retained on the floor, which was heavily coated with hard paraffin. Special precautions were taken to see that the water provided for drinking did not spill and so dilute the urine collected. This was accomplished by fixing a glass jar to the side of the cage as near to the top as possible, allowing just enough room for the monkey to pass its head to drink water in the standing position only. The depth of the jar and the level of the water in it were such that no spilling occurred even when the cage was shaken by the monkey, a common practice with these animals.

Urine and fæces were thus separately collected daily. The urine was received in a flask containing a little toluol. Half the quantity of urine collected daily was made slightly alkaline with a few drops of 10 per cent NaOH and evaporated to a syrupy consistency over a water-bath in a porcelain dish. The fæces were collected daily, dried at 80°C. and stored in a jar. The floor of the metabolic stand was washed with distilled water on the last day of the week. The washings were evaporated and the residue was added to the weekly collection of dried fæces. This procedure prevented decomposition of both urine and fæces prior to their final ashing.

The urine collected during the week was evaporated to dryness in a hot-air oven at 110°C. and finally over a sand-bath. It was then heated over a direct flame to char it. The final ashing was done in a furnace at 550°C. The weekly collection of dried fæces was ashed in the same manner.

The fluorine in urine and fæces was estimated by first distilling 0·5 g. of the ash by Willard and Winter's (1933) technique, and then treating the distillates by

the Sanchis' (1936) method. Aliquot parts of the distillates were taken and diluted, if required, to get the final readings of fluorine from 1 to 2 parts per million. This assured accurate calorimetric estimation of fluorine in the distillates. The total quantity of fluorine both in urine and fæces was then calculated.

In addition to the excretion of fluorine in urine and fæces, a few other bio-chemical investigations were done occasionally during the period of the experiment, e.g. sugar glycolysis and coagulation time of blood.

Radiological investigations.

These were undertaken to study the time of onset and the nature of the changes produced in bones and joints, as a result of ingestion of fluorides. *X*-ray photographs were taken at the commencement of the experiment and later after three and five months' intervals.

The course of intoxication in monkeys.

1. *Growth.*—Monkeys Nos. 1 and 2 (control group) kept up a progressive increase in weight throughout the course of the experiment. They were normally active. Monkeys Nos. 3 and 4, after an initial fall in weight when 20 mg. of fluorine were being given, recovered somewhat and remained stationary in weight during the period when 10 mg. of fluorine were being given. When this dose was reduced to 5 mg. they showed a definite tendency to gain in weight. Monkey No. 5 showed a rapid fall in weight during the 20 mg. and 10 mg. periods. When the dose was reduced to 5 mg. there was equally a sharp rise in weight. Monkey No. 6 remained almost stationary.

2. *General appearance and behaviour.*—Throughout the period of experiment, monkeys Nos. 1 and 2 remained active and healthy. In the animals receiving fluorides, no marked change either in appearance or behaviour was noticed during the first few weeks of observation. At about eight weeks after the commencement of the experiment, the monkeys receiving fluorides were found to be asthenic as compared with the normal monkeys and were less inclined to run or jump about. They were also anæmic. Subcutaneous fat was greatly reduced. The skin was loose and parchment-like and could be easily moved over the sub-cutaneous tissues. Falling off of hair was also noticed. These conditions were more marked in monkeys Nos. 5 and 6 than in Nos. 3 and 4.

Bony changes were first noticed in monkeys Nos. 5 and 6 at about three months from the commencement of the experiment. There were exostoses on the alveolar margin, and on the lower border of the mandibles. The long bones of the fore-arm and leg showed an irregular margin on palpation and thickening towards the distal ends. These changes progressed later during the period of observation so that they were readily noticeable to the naked eye. These changes were also noticeable in monkeys Nos. 3 and 4 but to a markedly less degree. In addition, monkeys Nos. 5 and 6 developed dactylitis of the fingers and toes—a condition which was most marked in No. 6. These had the appearance of tumours.

These changes were reflected in the movements of the respective monkeys. The gaits of Nos. 3 and 4 did not show any marked change from the normal, but in Nos. 5 and 6, the movements were definitely laboured, more so in No. 6, which could only crawl about. This animal showed occasional twitchings or fibrillations of the muscles of the body. Both the monkeys, Nos. 5 and 6, adopted a crouching attitude while resting. This was probably due to the increased curvature of the vertebral column which was also so rigid, that it was difficult to get the animals to lie flat on the table at the time of feeding. The radiological findings will be discussed later.

3. *Gastro-intestinal symptoms.*—During an initial period of five days when 20 mg. of fluorine were being given daily, all monkeys developed diarrhœa. After the dose was reduced to 10 mg. the condition improved, especially in the case of monkeys Nos. 3 and 4. Monkey No. 5, however, continued to pass semi-solid stools for a long time. In the eighth week of the experiment, symptoms of gingivitis were also noticed and since the animal was losing weight progressively, it was decided to introduce a minimal dose of vitamin C by giving it one banana. The monkey could still be maintained in a sub-scorbutic condition. After this, the diarrhœa ceased entirely and the general condition improved rapidly.

None of the monkeys excepting No. 4 showed any definite changes in teeth. It must be noted that monkeys Nos. 3 and 5 had erupted their permanent teeth prior to starting the experiment. Mottling of the enamel was, therefore, not expected to occur in these. During the period of observations, monkey No. 4 gradually dropped some of its deciduous teeth and developed two lower permanent central incisors, and later two upper central incisors. Mottling of the enamel of these was noted.

4. *Renal symptoms.*—The quantities of urine passed by monkeys Nos. 1, 3 and 5 were noted daily. These varied from day to day. In the earlier stages of the experiment, the degree of variation noted was almost the same in all the monkeys. From the eighth week onwards, however, when monkey No. 5 was showing symptoms of fluorine intoxication and had developed a scorbutic state, the variations noted in the quantities of urine passed became more pronounced. The quantity of urine excreted by monkey No. 5 gradually became less and less, while in the case of monkeys Nos. 1 and 3, the quantities remained at approximately their normal level. Probably, the kidney function was being impaired at this stage as was shown by the appearance of albumin in the urine. The albuminuria was, however, transient in the case of monkeys Nos. 3 and 5 but persisted in monkey No. 6 till its death from pneumonia, a few weeks later. Glycosuria was not observed in any of the monkeys during the period of observation.

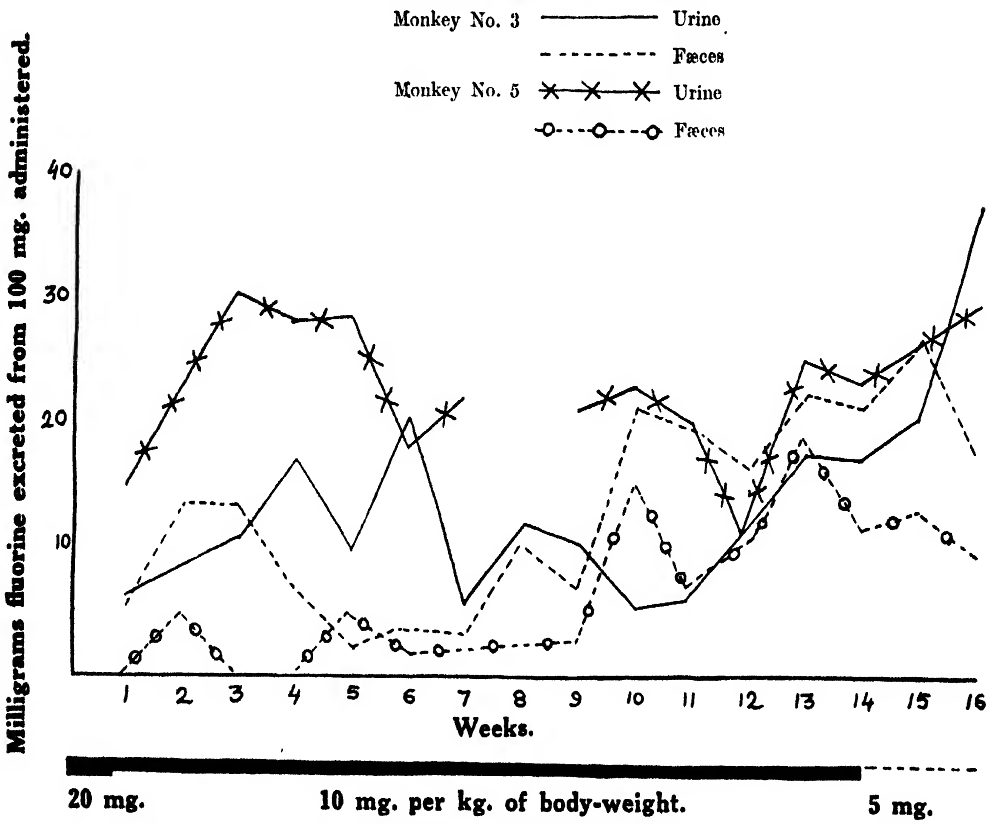
At the end of the fourth week from the commencement of the experiment, it was noted that the urine of monkeys Nos. 3 and 5 darkened on standing. This was not due to hæmaturia as tests for occult blood were negative. On investigation, it was found that the change in the colour was due to the presence of homogentisic acid. This condition persisted in all the monkeys so long as fluorine was being administered.

RESULTS OF BIOCHEMICAL INVESTIGATIONS.

(*a*) *Excretion of fluorine in urine and fæces.*—The quantities of sodium fluoride administered have already been referred to. The observations noted below on the excretion of fluorine in urine and fæces refer to the period when 10 mg. of fluorine per kg. of body-weight were being given, i.e. a period of about 16 weeks. The amounts of fluorine excreted in urine and fæces per week in terms

GRAPH 1.

Excretion of fluorine in urine and fæces.



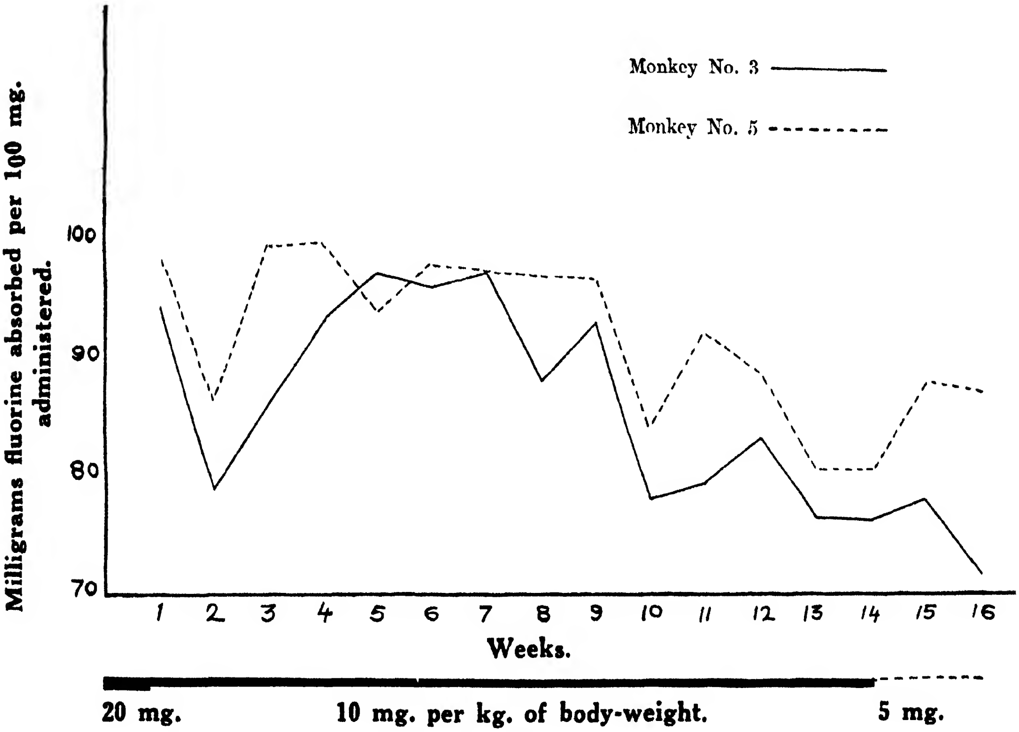
of 100 mg. ingested in the case of monkeys Nos. 3 and 5, are given in the accompanying Graph 1.

It will be seen that in monkey No. 3 for the first three weeks of observation, more fluorine was excreted in fæces than in urine. For the succeeding six weeks, more fluorine appeared in urine than in fæces. From the tenth week onwards, however, the ratio was again reversed.

In monkey No. 5, however, the excretion of fluorine in urine was very much higher than that in the fæces, throughout the period of the experiment. Only at about the twelfth week were the quantities of fluorine in the urine and fæces about equal.

(*b*) *Absorption and retention of fluorine in the system.*—The higher rate of excretion of fluorine in urine in the case of monkey No. 5. as compared with monkey No. 3, would appear to show that there was *initially* a greater absorption of fluorine into the system in the former than in the latter. In order to study this aspect of the question, the amounts of total fluorine absorbed week by week in both the

GRAPH 2.



monkeys, in contrast to the amounts finally retained, were calculated. For this purpose, the quantities of fluorine excreted in the fæces were subtracted from the total amount of fluorine administered to both the monkeys. The results obtained are expressed in Graph 2 in terms of 100 mg. of fluorine ingested by each animal.

It will be seen from this graph that the rate of absorption of fluorine has always been higher in monkey No. 5 than in monkey No. 3 and that the difference between the two was somewhat accentuated in the later stages of the experiment. The question, whether fluorine is excreted in the large intestines after absorption, will be referred to later.

Whether there are other channels of excretion of fluorine apart from urine and fæces yet remains to be ascertained. However, from the data furnished in Graph 1, it was possible to calculate the amount of fluorine finally retained by both the monkeys.

For this purpose, the period during which 10 mg. of sodium fluoride per kg. of body-weight were being given to both monkeys, was taken into account. Monkey No. 3 received this dose for a period of 16 weeks and monkey No. 5 for a period of 12 weeks. The amounts of fluorine received by monkeys Nos. 3 and 5 during this period were 3,272 mg. and 2,606 mg., respectively. The amounts finally retained were 71·9 per cent in the case of monkey No. 3 and 78·4 per cent in the case of monkey No. 5.

(*c*) *Blood glycolysis.*—Since fluorine is known to be an enzymatic poison and inhibits enzyme activity *in vitro*, attempts were made to study whether, as a result of concentration of fluorine in blood, glycolysis was being prevented.

The amounts of blood sugar in monkeys Nos. 1, 3 and 5 were estimated immediately after the withdrawal of blood and again four hours later. The tests were made at the commencement of the experiment and at three and six months' intervals. The results are given in Table I :—

TABLE I.

Estimation of blood glycolysis at periodic intervals.

Date.	Monkey number.	Blood sugar in mg.		Percentage of glycolysis.
		On withdrawal.	Four hours after.	
13-6-39 ..	1	136·0	54·3	60·0
	3	112·6	48·2	57·2
	5	110·2	43·3	60·7
26-9-39 ..	1	103·73	47·96	54·4
	3	107·3	45·15	53·4
	5	98·81	43·49	56·0
22-12-39 ..	1	76·0	26·32	65·4
	3	81·0	30·76	62·0
	5	74·3	28·48	61·7

It will be seen from the table that the amount of glycolysis was the same in the normal monkey No. 1 as in monkeys Nos. 3 and 5 which had received fluorides. The concentration of fluorine required to prevent glycolysis and inhibit other

enzyme activities has been stated by Ewig (quoted by Roholm, 1937, p. 63) to be m/100 of sodium fluoride. Apparently, this level of concentration of fluorine in blood had not been reached in the monkeys under experimentation.

Radiological findings.

Radiological investigations of all the animals, i.e., monkeys Nos. 1, 3 and 5 prior to fluoride administration, showed no abnormal appearance. These *X*-ray pictures served as standard normal appearances.

After fluoride administration radiological investigation was carried out at varying intervals, when monkeys Nos. 4 and 6 were also included. The following changes were noted :—

Monkey No. 5 showed the greatest degree of changes as seen in radiograms after five months of fluoride administration.

Head bones.—The skull in general and the teeth in particular showed increased density. The inner table of the skull appeared particularly thickened. The lower margin of the mandible showed nodular sub-periosteal deposits producing marginal irregularities and varying densities in the radiograms.

Bones of the trunk.

(*a*) *Spine.*—The dorsal and cervical regions showed no apparent changes. In the lumbar region, however, marked paravertebral deposits were seen, presumably in the lateral spinal ligaments. No osteophytic outgrowths from the vertebræ were seen, although the vertebræ showed increased density.

(*b*) *Ribs.*—A few, particularly the lower ones, showed increased density along the margins.

(*c*) *Pelvis.*—The iliac crests, the lower margins of the ischial and pubic rami, the margins of the obdurator foramen and acetabula showed dense sub-periosteal deposits.

(*d*) *Shoulder girdle.*—Around the glenoid fossæ nodular deposits were seen.

Long bones.

(*a*) *Humeri.*—The inner aspects of the upper half of the humeri showed sub-periosteal deposits.

(*b*) *Femora.*—The intertrochanteric ridge and upper part of the shafts showed periosteal densities.

(*c*) *Radii and ulnæ.*—The upper and lower ends of the ulnæ showed sub-periosteal deposits. This was seen to a lesser degree in the radii which, at their lower ends, showed a fusiform appearance on account of lifting of periosteum on either side. Along the margins of the interosseous membranes, there were what appeared to be commencing deposits. Similar changes, but less marked, were obtained in the long bones of the posterior extremity.

Other bones.—The metatarsal and metacarpal bones showed sub-periosteal deposits with thickening of the periosteum.

Monkey No. 3.—This animal showed similar changes to those described above, except that these changes were less marked generally. The vertebral column showed the least change. The radii and ulnæ, however, showed marked changes. It may be mentioned that in this animal, changes occurred later than in monkey No. 5.

Monkey No. 4.—This animal belonged to group B, i.e. the same as monkey No. 3 but it showed no marked radiological changes. At only a few sites, subperiosteal deposits were noted. The changes in the lumbar vertebræ were of an incipient character.

Monkey No. 6.—This animal belonged to group C, i.e. the same as monkey No. 5. The reactions of the bones of the extremities revealed several nodular periosteal deposits. The animal died. A complete examination of the skeleton revealed lesions shown in the photograph. It would appear that the nodular thickenings in the skeleton were most marked at the sites of muscular attachments.

These changes are illustrated in Plates XI to XIV.

Discussion.

Attempts have been made by several workers to produce symptoms of chronic fluorine intoxication in experimental animals. Rats were commonly employed for the purpose although some used dogs, pigs and sheep for the study. Sodium fluoride was the common fluorine compound used which was administered mixed with food. The symptoms noted varied in different animals. Apart from changes in the teeth, rats showed, generally, a retardation of growth and no pronounced changes in bones, while in pigs thickening of mandibles and exostoses on long bones were often noted. The time required to produce such changes also varied in different animals and depended on the fluorine compound used. When comparatively insoluble compounds were used, e.g. calcium fluoride, sodium-aluminium-fluoride, sodium silico-fluoride, a much longer period of administration was required to produce the changes noted than was the case when sodium fluoride was used. These findings have been summarized by Roholm (*loc. cit.*).

The primary object of this investigation was to ascertain the action, if any, of vitamin C intake in the diet on the production of symptoms of chronic fluorine intoxication in monkeys. The biochemical data recorded in this paper, however, relate to observations on two monkeys only. Owing to the nature and volume of technical work involved, it was not possible to extend the detailed biochemical tests to all the monkeys under observation. Since individual variations have to be taken into account in assessing the results, no final conclusions can be drawn on the evidence presented in this paper. The results are discussed subject to this limitation.

It was found that fluorine appeared in the urine and fæces of experimental animals from the commencement of the experiment. This observation is contrary to that recorded by Brandl and Tappeinur (quoted by Roholm, 1937, p. 106) who did not find any fluorine either in urine or fæces of a dog during the first three

weeks of administration in doses varying from 0·1 g. to 1 g. of sodium fluoride daily. These results are not understood, especially when the same authors found fluorine in urine immediately after subcutaneous injection, unless the use of animals of different food habits is the explanation of this obvious discrepancy.

That the rate of fluorine excreted in urine was greater in monkey No. 5 than in monkey No. 3 has already been described. Since fluorine must be absorbed into the system before it can be excreted in urine, the higher rate of excretion of fluorine in monkey No. 5 suggests a higher degree of 'fluoræmia' in this animal than in monkey No. 3.

It thus appears that the greater absorption of fluorine in monkey No. 5 was probably facilitated by the absence of vitamin C in the diet.

The presence of homogentisic acid in the urine is suggestive of an interference with the metabolism of phenyl alamine and tyrosine. The significance of this will require further investigation.

From the radiological findings in all the monkeys it will be seen that in monkeys Nos. 5 and 6, which were deprived of vitamin C in the diet, the changes were much more pronounced than in monkeys Nos. 3 and 4 which received a liberal supply of vitamin C. Absence of this vitamin in the diet, therefore, not only promotes greater absorption but probably enhances the deposition of fluoride salts in bones as shown by their greater densities. When it is remembered that the absence of vitamin C causes special damage to capillary endothelium and that the periosteum is a highly vascular tissue, the deposition of fluoride salts at such sites is readily understood. It is significant that the maximum deposits occurred at sites involving the greatest movements. It would, therefore, appear that the absence of vitamin C in the diet favours the production of symptoms of chronic fluorine intoxication.

The hypothesis that the absence of vitamin C in the diet can cause diffuse periostitis in children has been put forward by Ellis (1939). He suggests that periostitis may have been due to a low grade of infection complicating a state of vitamin C deficiency not severe enough to cause scurvy.

It must be stated here that the radiological changes produced in monkeys were not entirely analogous to those observed in human beings. In the latter, the condition was marked by osteophytic outgrowths and calcification of tendinous attachments of ligaments. In monkeys, the changes were mainly of the nature of diffuse periostitis. As regards the vertebral column, the changes were probably the same in both cases. It may be stated also that changes in both appeared to be of the osteosclerotic type. In comparing the changes produced in experimental animals and human beings, however, several points, e.g. the dosage of fluoride salts, the period of exposure to intoxication and the mineral content of the diet, have to be considered. Smaller doses administered over long periods might have given rise to an entirely different picture.

In the majority of experimental animals, changes of the osteoporotic type have usually been observed. The apparent osteosclerotic changes noted in

Explanation of Plate XI.

Appearance of monkey No. 5 prior to the commencement of experiment (normal).

PLATE XI.

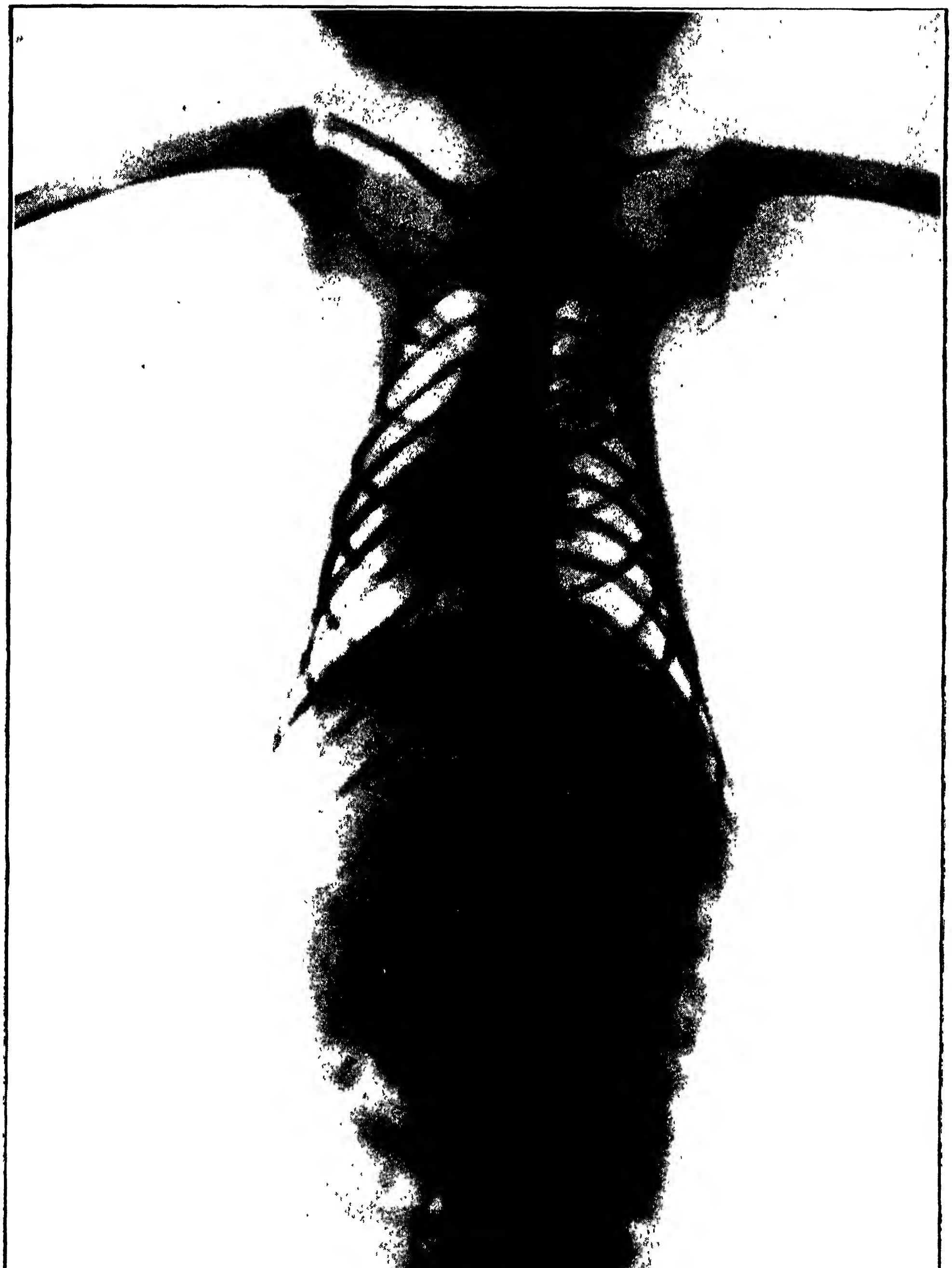


PLATE XII.

Explanation of Plate XII.

Monkey No. 5, five months after commencement of experiment—(*i*) periosteal deposits on the shafts of the humeri and near the glenoid fossæ; (*ii*) increased density of lumbar vertebræ and paravertebral deposits.

EXPLANATION OF PLATE XIII.

Changes in the bones of forearm and hand of monkeys Nos. 3, 5 and 6.

A. Normal appearance of monkey No. 5.

B. Monkey No. 5, five months after commencement of experiment, showing diffuse periosteal deposits along the ulna particularly near the interosseous membrane and fusiform swelling at the lower end of the radius.

C. Monkey No. 3, five months after commencement of experiment, showing dense periosteal deposits along the entire length of both radius and ulna.

D. Monkey No. 6, showing intense periosteal deposits along the entire surface of radius and ulna and, in addition, nodular thickenings. The metacarpal bones and phalangeal bones show periosteal nodular swellings.

PLATE XIII.

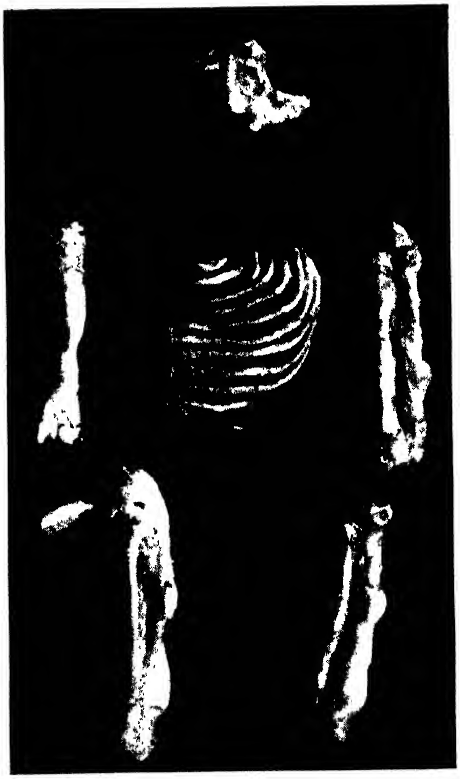
A B C D

PLATE XIV.

A.

B.

C.



D.

Explanation of Plate XIV.

A. Skiagram of skull of monkey No. 5, before commencement of experiment (normal).

B. Skiagram of skull of monkey No. 5, five months after commencement of experiment showing thickening of the inner table and general density. Note also the irregular margin of the mandible.

C. Monkey No. 6, showing (*i*) crouching attitude and puffiness of face, (*ii*) exostosis on the lower margin of the mandible (the upper nodular swelling is due to food in the pouch) and (*iii*) irregular outline of limbs due to nodular swellings.

D. The appearance of bones of monkey No. 6, (*i*) exostoses on the mandible and the long bones are well seen, (*ii*) swellings at sites of muscle attachments and (*iii*) irregular margin of the ribs.

monkeys might have been due to the high mineral content provided in the diet. These points would require further investigation.

Summary.

Experiments were undertaken to produce symptoms of chronic fluorine intoxication in monkeys (*M. radiata*) by administration of sodium fluoride and to study the action, if any, of vitamin C in the diet. The absorption and excretion of fluorine in these animals were studied. Monkeys which did not receive vitamin C in the diet showed greater absorption of fluorine and greater excretion in urine as compared with monkeys which had received a liberal supply of vitamin C. Glycolysis *in vitro* was not materially affected in the two groups of monkeys. Radiological changes were also more marked in vitamin C deficient animals. The significance of these findings is discussed.

Addendum.

Monkeys Nos. 3 and 6 died during the course of the experiment. The various organs did not reveal any abnormal changes except a slight hyperplasia of the thyroid in monkey No. 3, and a cloudy swelling of the kidneys in monkey No. 6. The concentration of fluoride salts in several organs is being determined. After the cessation of the experiment, the administration of fluorine to monkeys Nos. 4 and 5 was discontinued. Examination of urine six weeks later still showed the presence of fluorine, 9 mg. per week in both cases. These observations are being continued and extended.

Acknowledgments.

This research was financed by a grant from the Indian Research Fund Association. Our thanks are due to Captain T. W. Barnard, Director, and Dr. M. J. S. Pillay, Medical Superintendent of the Barnard Institute of Radiology, for their help in the radiological investigation of our material. We are also indebted to Dr. P. Rama Rao for taking *X*-ray pictures of monkey No. 6. Our thanks are also due to Dr. V. Krishnamurti for his technical help in the conduct of the experiments.

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FURTHER INVESTIGATIONS ON THE TRANSMISSION OF KALA-AZAR.

Part I.

THE MAINTENANCE OF SANDFLIES *P. ARGENTIPES* ON NUTRIMENT OTHER THAN BLOOD.

BY

R. O. A. SMITH,

K. C. HALDER,

AND

I. AHMED.

(From the Kala-azar Inquiry under the Indian Research Fund Association, Darbhanga Medical School, Bihar.)

[Received for publication, May 24, 1940.]

THE discovery of the ex-flagellation of *L. donovani* in the gut of the sandfly *P. argentipes* (Knowles, Napier and Smith, 1924), and the further development of the flagellate infection anteriorly resulting in an invasion of the pharynx of the fly (Christophers, Shortt and Barraud, 1925), led to a large number of feeding experiments to prove the transmission of kala-azar by the bite of this sandfly. The method generally adopted for re-feeding and keeping these flies under laboratory conditions was that recommended by Shortt, Barraud and Craighead (1926) and consisted, briefly, in keeping each fly which had fed on a kala-azar patient in a separate tube, the plug of which was moistened just enough not to cause a film of moisture to settle on the inner surface of the tube, and the tubes placed in an incubator at a temperature of about 28°C. Kept in this manner the blood meal was digested in three or four days, and a good proportion of the flies were found to have oviposited. The flies which oviposited were offered a second blood meal, and the same routine followed till the death of the fly when it was dissected

and examined. The results obtained by this method were sufficient to obviate the necessity for the investigation of substances other than blood on which sandflies would feed and thrive; perhaps the name Phlebotomus itself was also in some manner responsible for the impression that these insects could not live without blood, and even attempts at the artificial feeding of sandflies involved the use of blood in some form or other.

In all transmission experiments by the bites of *P. argentipes* conducted up to the present, the flies used for the purpose were maintained on successive blood meals. The first and usually the second feed also were given on a kala-azar patient, while the third and subsequent feeds were on experimental animals. It was assumed that not till the flies were ready for a third feed, which was about six or seven days after the first blood meal, were flagellates present in sufficient numbers at the 'anterior station' to be inoculated into a victim at the bite of the fly.

The uniformly negative results obtained during the first few years when mice, men and hamsters were experimented on, and the paucity of successful transmissions to such susceptible animals as hamsters in later years, led certain workers to doubt the rôle of the sandfly in the transmission of kala-azar, and to investigate methods of infection other than by an insect vector.

The fact that hamsters could be infected by the oral route (Shortt, Craighead, Smith and Swaminath, 1928) and reports of the finding of parasites in the fæces of kala-azar patients (Shortt, Smith, D'Silva and Swaminath, 1929), as well as in the nasal and pharyngeal secretions (Forkner and Zia, 1934) was evidence in support of such an hypothesis. Others were of opinion that a study of the bionomics of *P. argentipes*, of which so little is known, would most probably lead to the incrimination of this insect as the vector; for not only was the circumstantial evidence in support of the sandfly transmission of kala-azar very strong and in complete accord with our present knowledge of the epidemiology of the disease, but more especially because a few successful transmissions had already been obtained by the bites of these flies.

When research on the transmission problem was renewed last year it was the opinion of the senior author (R. O. A. S.) that a change from the methods practised previously was very essential before any advance in our knowledge of the subject could be expected. Variations in the technique of breeding the flies in the laboratory as well as in the temperature at which the flies were kept had already been tried and found to be of no avail; the possibility that a change in the food of the flies might produce some favourable result was therefore investigated. It was reported by Smith (1935) that *P. argentipes* could be maintained successfully under laboratory conditions, for relatively long periods if they were fed on a 5 per cent solution of glucose. This was very simply accomplished by substituting the watch-glass containing cotton-wool moistened with water, as was usually placed in the cages with the flies, by one with the pad of cotton-wool soaked in sterile glucose solution. The rapidity with which bacteria grew in the glucose and infected the flies was, however, a serious drawback to this method of maintaining them. Bacterial infections either killed the flies themselves or caused the death of the flagellates contained in their mid-guts. The method was therefore not suited

for keeping sandflies for transmission experiments. The fact that sandflies could subsist on nutriment other than blood led to the testing of the value of various fruit juices as a substitute for glucose and it was soon evident that, while slices of ripe banana were distinctly unfavourable to the longevity of *P. argentipes*, they did very well on apple but best of all on raisins, and like mosquitoes they survived for relatively long periods, both before and after a blood meal on this diet. The method employed was as follows :—

'To avoid unnecessary contamination of the chimney, on the third day after a feed on a kala-azar patient when practically the whole blood meal had been digested and excreted, the flies are introduced into a globular chimney which is closed at one end with a piece of muslin, and plugged at the other with cotton-wool. In the centre of the plug was inserted a specimen tube about three-quarters to seven-eighths of an inch in diameter, filled almost to the top with cotton-wool, leaving a shallow depression to contain the raisins. The rim of the tube was adjusted very slightly above the level of the plug. The raisins are prepared by washing them in running water for about half an hour and then scalding in boiling water for a minute. To obtain the best results with infected flies bruised raisins and those that emit an odour of acid fermentation should not be used, also the raisins require to be changed every day, as the temperature and humidity at which it is necessary to keep these flies causes a rapid growth of moulds on the raisins in a little over 24 hours and sometimes in less.

Before the flies are introduced into a chimney so prepared the cotton-wool plug is moistened at the periphery only. Considerable numbers of ova are found deposited on the damp part of the plug by the fourth or fifth day and if required for breeding purposes the plug is with advantage substituted at this stage for a fresh one. Once the flies had oviposited the absence of blood meals precluded the development of further batches of ova.

From 40 to 50 flies are conveniently housed in a chimney of about 700 c.c. capacity.

Small muslin cages have been found as suitable or better than chimneys for housing the flies, as the survival rate has very often been found to be greater in cages than in chimneys at the end of a ten-day period. If a cage is used a watch-glass containing a pad of damp cotton-wool should be introduced as well as one for the raisins. This method is, however, not the best if the flies are to be used for transmission experiments, as we have reason to believe that flagellate infections develop better in flies housed in chimneys, probably because a more constant humidity is obtained in a chimney than in a muslin cage.'

This work was conducted between June and October and the chimneys and cages with the flies were kept in an incubator which was unregulated and in which during the period mentioned above there was very little variation from the optimum of 28°C. required for the best results from this species.

In the transmission experiments conducted with these flies, they were kept till the tenth day in the manner described above before being given a second blood

meal on an experimental animal. Flies of this age are the equivalent of fourth or fifth feed flies kept according to the old method of successive blood meals, and flagellate infections were expected to be well advanced. Flies which refused to feed on one day were replaced in a chimney with raisins for a further 24 hours, when many of them were found to take a meal. This procedure was followed till the batch was exhausted.

The method employed in previous years aimed at keeping *P. argentipes* as much under natural conditions in the matter of food and temperature and humidity as was possible in a laboratory, on the assumption that by such means would the best results be obtained.

The change therefore to the present method of feeding the flies may seem unnatural and unlikely to yield satisfactory results; flagellate infections however have been found to develop uninterruptedly in the flies and it may even be claimed that a larger proportion of flies are found with heavier infections by this method than was seen when they were given repeated blood meals. It is not possible to produce figures in support of this contention as no records of the intensity of infections in flies were kept previously and it was not possible to maintain a parallel series on blood meals on this occasion. The opinion is, however, that of the senior author who has dissected very large numbers of sandflies fed on repeated blood meals and has dissected all the flies in this series.

It has been estimated that by this method an average of 50 per cent of flies which take a first blood meal on a kala-azar patient are available at the end of ten days for delivering infective bites. Of 1,566 flies fed and kept on raisins in September when the general atmospheric temperature and humidity conditions were most favourable 855 were alive on the tenth day. From figures supplied by the senior author to determine the duration of the life of *P. argentipes* in the laboratory Raja (1936) estimated that 74·33 per cent of flies died about the sixth or seventh day when maintained on successive blood meals, and about 15 per cent only survived till the tenth day. The advantages to be derived from the new method are therefore obvious, for in addition to larger numbers of more heavily infected flies being available for transmission experiments there is also a considerable saving of time and labour when large batches of flies have to be handled.

Wild flies may be maintained on raisins in the same manner as laboratory-bred ones, but whether they take any food other than the blood under natural conditions is not known. The fact that *P. argentipes* are most prevalent under rural conditions where the opportunities for imbibing fruit and plant juices are present practically throughout the year would favour such a possibility. The detection of breeding sites of this species in soil protected by heavy undergrowth is evidence that they do resort to vegetation for some time at least. It must, however, be stated that repeated attempts to capture flies from such situations have up to now not yielded any encouraging results.

A test of the infectivity of flagellates from sandflies fed on raisins has been made by inoculating mice both intraperitoneally and subcutaneously with such

flagellates. The examination of these animals after a sufficient incubation period, will serve as an indication of the results to be expected from the feeding experiment with the flies.

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FURTHER INVESTIGATIONS ON THE TRANSMISSION OF KALA-AZAR.

Part II.

THE PHENOMENON OF THE 'BLOCKED' SANDFLY.

BY

R. O. A. SMITH,

K. C. HALDER,

AND

I. AHMED.

(*From the Kala-azar Inquiry under the Indian Research Fund Association, Darbhanga Medical School, Bihar.*)

[Received for publication, May 24, 1940.]

THE feeding of infected sandflies on hamsters and mice in experiments to prove the transmission of kala-azar by the bite of the sandfly *P. argentipes* was usually carried out by laboratory assistants under the supervision and direction of a member of the senior staff. The flies to be fed were placed in tubes, one to three or four to a tube, which was inverted over the shaved abdomen of the victim. The tube was held in position by the assistant till such time as the fly obtained a meal of blood or it was assumed after a reasonable length of time that the fly was not prepared to feed. Both the fly and the animal were under the close observation of the assistant throughout the whole operation.

The senior author (R. O. A. S.) was closely connected with this aspect of the work since the inception of experiments to determine the rôle of the sandfly in the transmission of kala-azar and two of the assistants at present entrusted with the work of feeding sandflies have had many years' experience in the handling of these insects at the School of Tropical Medicine, Calcutta. A report from one of them therefore that certain flies were found making persistent efforts to obtain a blood meal after puncturing the skin of the animal and failing to succeed was carefully

investigated, as such an observation had never been made before. Reports to the effect that certain flies took an unusually long time to obtain a meal of blood were not uncommon previously, as well as the fact that certain flies took a relatively very small amount of blood ; but such flies unfortunately were not singled out for special attention, and whether they were heavily infected individuals or merely feeble ones which were unable to collect a full blood meal easily for reasons other than that their passages were obstructed with masses of flagellates cannot now be ascertained.

The separation and examination of flies reported to have made persistent efforts to obtain a blood meal, sometimes for as long as ten minutes, without succeeding in doing so, revealed the fact that they were almost invariably heavily infected individuals. These flies were carefully dissected and in those instances when the head capsules of some of them had been successfully removed it was seen that the proventricular fold was almost completely obliterated and the œsophagus greatly distended with flagellates ; the extent to which the pharynx was involved could not, however, be determined in a dissected specimen owing to the opacity of the chitinous walls of that organ ; but on more than one occasion when freshly dead flies were dissected, elongated and very actively motile flagellates were seen escaping from the anterior end of the pharynx when its attachment to the buccal cavity was severed. These features have also been observed in the dissection of flies which showed no signs of obstruction, to the flow of blood into the midgut, but not it is thought to such a marked extent.

The condition was therefore regarded as one of 'blocking' and similar to that occurring in fleas infected with *Pasteurella pestis*.

The first description of 'blocking' in sandflies was made by Shortt, Barraud and Craighead (1926). Describing the life history and morphology of *H. donovani* in sandflies as seen in sections of infected flies they state, 'As time proceeds there is a continuous extension forwards of the massive growth in the pharynx until the lumen of that organ for the greater part of its length, and sometimes in its entire length up to its junction anteriorly with the buccal cavity, becomes blocked with a solid plug of flagellates recalling the condition to be found in plague fleas where the œsophagus may be actually blocked by plague bacilli'.

On this occasion flies were diagnosed 'blocked' on being found unable to take a meal of blood, after piercing the skin of the victim and endeavouring to do so.

'Blocking' in sandflies may be partial or complete. In the partially 'blocked' flies a very small quantity of blood may be seen with the naked eye, but more often the aid of a lens is required to detect the minute quantity of blood seen as a pink discoloration about the thorax of the fly. No blood can be seen in the abdomen. In the completely 'blocked' fly no evidence of blood can be detected with a lens nor has any been seen in the midgut at dissection.

The condition was first noted on the 10th of August and not detected again after the 2nd of October. This interval coincides with the post-monsoon period when atmospheric temperature and humidity is generally at its highest and

incidentally when this species is most prevalent under natural conditions. Of 225 known positive flies dissected during the course of these experiments 58 were diagnosed 'blocked' and separated for special examination. The dissection of these flies as they died gave the following results :—

Heavily infected with flagellates	49
Negative—no flagellates seen	2
Fly decomposed—no result possible	7

In nearly 22 per cent of infected flies complete obstruction to the midgut was found. As however 'blocked' flies do not live for longer than a day or two, and since the phenomenon would depend on the number of L. D. bodies ingested by the fly at its first feed, and develop earlier or later as particular flies obtained larger or smaller numbers of parasites, the proportion that will show the symptom of obstruction would depend not only on the temperature and humidity, but also on the time elapsing from the first feed to the testing of the flies for the condition, by the offer of a second blood meal.

The 'blocked' flies, as they were detected each day, were housed together in one large chimney in order to keep them alive as long as possible for feeding on the experimental animals, and no details regarding the duration of life and other particulars of individual flies have been made up to date. The following history of three flies is mentioned to give an indication of what generally transpires once the condition of 'blocking' is established in the fly :—

Three flies from a batch of 46 given one morning for the first infective feed were found 'blocked'; they were placed in separate tubes and after a sufficient interval again offered a feed on a clean hamster. They pierced the skin of the animal and attempted for approximately ten minutes each to collect a meal of blood. The examination of the flies at the end of the period revealed the fact that no blood had been taken. They were then placed in a chimney with raisins till the next day. One fly was found dead the next morning, and on dissection proved to be heavily infected with flagellates. The two remaining flies were offered a feed on a hamster, but though they made attempts to feed their efforts were not as persistent as on the previous day. The next day both flies were found dead and on dissection proved to be heavily infected.

In former experiments when *P. argentipes* were maintained on successive blood meals, these were offered as soon as the previous meals taken by the flies were seen to have been digested and, as was usual, that the flies had oviposited. Depending on the temperature and humidity the first, second, third, fourth, and subsequent feeds were taken on the first, third, sixth, eighth or ninth and tenth or eleventh day of the life of the fly approximately, and although heavy flagellate infections were frequently found complete obstruction to the flow of blood to the midgut was either absent altogether or if it did occur was so rare that it was not recognized. According to the present routine the flies after a first feed on a kala-azar patient are kept on a diet of raisins till the tenth day before they are offered a second blood meal on an experimental animal.

Flies which feed a second time are tubed separately and given subsequent feeds when the previous meals have been digested. The condition of ' blocking ' has been observed only among the flies which are being offered a second blood meal, which as stated above is given about the tenth or eleventh day after the first feed. The condition has not been found among the flies of this series which are being offered third, fourth and subsequent feeds, although among them heavily infected individuals have been found on dissection. If ' blocking ' was exclusively the result of a progressive multiplication of flagellates, it should have been detected oftener (or at least in some instances) in flies at the third, fourth or fifth feed than at the second, as the former had from two to six days longer to develop larger numbers of flagellates. That this is not the case is suggestive that some at present unknown factor is necessary in addition to a heavy flagellate infection to produce the phenomenon, or that some factor which prevents ' blocking ' is in operation when sandflies are fed on repeated blood meals.

The inability of ' blocked ' flies to obtain blood after piercing the skin of the animal is, we think, due to the enormous number of flagellates in the œsophagus rather than in the pharynx of anterior end of the midgut. The significance of the phenomenon cannot be assessed till the results of the present series of transmission experiments are available. It is, however, not unreasonable to expect that ' blocked ' flies would make more vigorous attempts to satisfy their hunger and thereby cause the detachment and inoculation of larger numbers of flagellates than ordinarily infected flies which apparently have little or no obstruction to the free flow of blood into their midguts. The results to be expected from the bites of ' blocked ' flies would therefore seem to be better than from those which do not exhibit this symptom.

We take this opportunity to express our thanks to Mr. J. A. Dey, the assistant, whose observation led to the recognition of the phenomenon of ' blocking '.

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Ind. Jour. Med. Res., **28**, 2, October, 1940.

FURTHER INVESTIGATIONS ON THE TRANSMISSION OF KALA-AZAR.

Part III.

THE TRANSMISSION OF KALA-AZAR BY THE BITE OF THE SANDFLY *P. ARGENTIPES.*

BY

R. O. A. SMITH,

K. C. HALDER,

AND

I. AHMED.

(*From the Kala-azar Inquiry under the Indian Research Fund Association, Darbhanga Medical School, Bihar.*)

[Received for publication, May 24, 1940.]

THE first successful transmission of *L. donovani* by the bite of the sandfly *P. argentipes* was obtained in a hamster in 1931 (Shortt, Smith, Swaminath and Krishnan, 1931). The animal in question was one of two which had survived an incubation period of about 17 months before it was sacrificed and examined. In previous experiments many of the animals exposed to infection had died or been sacrificed after shorter incubation periods. It was suggested, therefore, that one of the conditions for a successful transmission was a prolonged incubation period and further experiments to improve on and confirm this result were undertaken without any significant change in the methods practised in feeding and keeping the flies; but as far as possible the animals experimented on were kept for a year and upwards before they were sacrificed for examination. The transmissions obtained in 1933, two out of 28 (Napier, Smith and Krishnan, 1933), and in 1935, one out of 16 (Smith, Lal, Mukerjee and Halder, 1936), while confirming the

fact that flagellates are injected at the bite of an infected fly, were not quite the results expected if *P. argentipes* was to be incriminated as the vector of kala-azar under natural conditions. The reason why so many hamsters (animals known to be very susceptible to Leishmaniasis) failed to show infections after being bitten by relatively large numbers of infected flies could not be satisfactorily explained; and, further, no great hope of success could be expected from experiments to transmit the disease to a human being, a relatively insusceptible subject, if but only one out of 10 or 20 very susceptible animals could be infected by multiple bites from infected flies.

When research on the problem of transmission was resumed in 1939, a change in the method of feeding and keeping the flies was made as described in Part I of this series (*see* page 575, this issue). The flies after a preliminary feed on a kala-azar patient were maintained till the tenth day on a diet of raisins and then offered a second blood meal on an experimental animal.

Five hamsters and ten mice were exposed to the bites of such flies, but as two mice died very shortly after being placed under experiment they have been excluded from this series. In the Table are given the results obtained.

It is very satisfactory to note that seven out of the 13 animals placed under experiment have been proved to be infected, and especially that two mice are included among the successful ones. Mice are considered to be relatively resistant to infections with Leishmania, and the fact that two, which had no great number of known positive feeds, showed infections leads us to think that some at least of the others which had larger numbers of known positive feeds would have been found infected had they survived a little longer, or had cultures of spleen and liver tissue been possible.

Hamster No. 5 died of a large abdominal tumour which had involved the stomach and spleen, and the detection of L. D. bodies in the smears of the liver and bone-marrow six weeks after the animal was placed under experiment, as well as the very heavy infection found in hamster No. 8, after an incubation of ten weeks only, were indications that no prolonged incubation would be necessary on this occasion before the examination of the experimental animals could be undertaken. The longest incubation of any animal in this series was in respect of mouse No. 1, and was a little more than seven months. L. D. bodies were found in the spleen smears and flagellates in the cultures.

It would appear that complete obstruction to the passage to the midgut of the fly is not an essential factor in transmission. The two mice found infected had, as far as is known, no bites from 'blocked' flies. But that 'blocked' flies do inject many parasites in their efforts to obtain a meal of blood is exemplified in the case of hamster No. 11 which was bitten by 'blocked' flies only; the liver puncture material from this animal revealed very large numbers of L. D. bodies. Probably there are various degrees of 'blocking' and certain flies which are incompletely 'blocked' and able to take a meal of blood deliver as large doses of flagellates at their bites, as those which are completely 'blocked' and unable to obtain any blood.

Table.

Serial number and nature of animal.	Date of commencement of experiment.	Total number of feeds by *P. argentipes*.	Number of flies known to be not infected.	Number of flies known to be infected.	Number of 'blocked' flies.	Date of termination of experiment.	Nature of termination.	Result.	Remarks.
Mouse No. 1 ..	30–6–39	89	33	10	0	10–2–40	Sacrificed	Positive	L. D. bodies in smears of spleen and flagellates in culture.
,, No. 2 ..	2–7–39	96	42	18	0	11–12–39	Found dead	Negative	Result inconclusive; no culture possible.
,, No. 3 ..	4–7–39	88	46	12	0	10–2–40	Sacrificed	Positive	By culture only; no L. D. bodies seen in smears of liver or spleen.
,, No. 4 ..	10–7–39	135	57	9	2	23–11–39	Found dead	Negative	Result inconclusive.
Hamster No. 5	3–8–39	253	91	32	3	16–9–39	,, ,,	Positive	Death caused by large abdominal tumour. L. D. bodies in smears of liver and bone-marrow.
Mouse No. 6 ..	10–8–39	189	83	19	0	5–11–39	,, ,,	Negative	Result inconclusive.
,, No. 7 ..	9–8–39	174	64	22	7	14–11–39	,, ,,	,,	,, ,,

Note.—Mice Nos. 3 and 6 were bitten by flies at the third and subsequent feeds only.

TABLE—*concld.*

Serial number and nature of animal.	Date of commencement of experiment.	Total number of feeds by *P. argentipes*.	Number of flies known to be not infected.	Number of flies known to be infected.	Number of 'blocked' flies.	Date of termination of experiment.	Nature of termination.	Result.	REMARKS.
Hamster No. 8	12-8-39	114	35	19	10	29-10-39	Found dead	Positive	Killed accidentally. Numerous L. D. bodies in smears of liver and spleen.
Mouse No. 9 ..	19-9-39	294	157	10	0	6-1-40	,, ,,	Negative	Result inconclusive.
Hamster No. 10	26-8-39	78	27	6	4	9-2-40	Sacrificed	Positive	Numerous L. D. bodies in smears of liver and spleen.
,, No. 11	13-9-39	24	2	20	20	8-2-40	..	,,	Hamster not sacrificed. Numerous L. D. bodies in liver puncture material.
Mouse No. 13 ..	18-9-39	140	53	11	*	11-12-39	Found dead	Negative	* This animal was used to test for 'blocked' flies before they were fed on hamster No. 11. Result inconclusive.
Hamster No. 14	26-9-39	116	53	10	3	9-2-40	Sacrificed	Positive	Fair numbers of L. D. bodies in smears of liver and spleen.

Note.—Mouse No. 9 was bitten by flies at the third and subsequent feeds only.

The factor responsible for the success in this series of transmission experiments is still to be sought. Was transmission effected as the result of an increase in the virulence of the parasites or to larger doses of flagellates inoculated at the bites of the flies? If in the virulence of the parasites, were the patients used for infecting the flies harbouring a more virulent brand of *L. donovani*, or was the change in the food of the flies from blood to fructose responsible for an increased infectivity of the flagellates?

Commenting on the first successful transmission obtained in 1931, Shortt *et al.* state that the fact that only one out of 42 hamsters experimented on got infected was due to the low infection rate by the bite of *P. argentipes*, which also explained the slow spread of kala-azar during inter-epidemic periods; but that during an epidemic when there was a rapid succession of passages from man to fly and vice versa, it was possible that there would be an increase in the virulence of the parasites quite out of proportion to that possessed during inter-epidemic periods. It may, however, be mentioned that kala-azar has been known to be endemic in the district of Darbhanga for over 20 years, and though at the present time there are certain areas where a 'flare-up' of the disease has occurred following localized epidemics of malaria, in no instance were patients imported from such areas for infecting the flies for these experiments. The cases used were those voluntarily attending the hospital from villages mostly in the vicinity of the town of Darbhanga.

This fact does not of course rule out the possibility that the patients were responsible for infecting the flies with a more virulent brand of *L. donovani*, and therefore for the successful transmissions.

Certain anomalous results in connection with the feeding of sandflies on repeated blood meals, however, lead us to think there may be a different explanation for the transmissions. As early as 1926 it was remarked by Christophers, Shortt and Barraud (1926) that the proportion of infections encountered in twice-fed flies was rather small considering that such flies have had twice the chances of flies fed only once of becoming infected. This has been the experience subsequently of other workers in this field of research. Shortt, Barraud and Craighead (1926) produce figures to show that when flies are fed on kala-azar cases the most favourable conditions as regards the second feed for the production of a very heavy infection with flagellates, is that it should be either on the same kala-azar case as the first or on an animal. No satisfactory reasons for such findings have yet been offered.

Also the observation by Krishnan (1937) that the action of fresh normal or kala-azar serum on *H. donovani* results in the destruction of these flagellates by lysis is suggestive that successive blood meals, such as were given the flies in previous years, were not conducive to the optimum multiplication and development of *H. donovani* in *P. argentipes*. The detection during these feeding operations when the flies were maintained on raisins of a good proportion of them so heavily infected that total obstruction to the flow of blood into the midgut was present, would support such a contention. For the same reason it may be inferred

that the flies fed on raisins, whether they showed the phenomenon of 'blocking' or not, were generally more heavily infected at the critical points, and consequently that larger doses of parasites were inoculated when they fed on the experimental animals.

The fact that two mice were found to be infected, as well as the intensity of the infections in the hamsters after comparatively short incubation periods leads us to infer that there was also an increase in the virulence of the parasites compared with former transmission experiments. It is unfortunate that nine out of the ten mice artificially inoculated with flagellates from one sandfly each, as a test of the infectivity of the flagellates from sandflies fed on raisins, died before an adequate incubation period had elapsed, and no parasites were detected in the smears of their liver, spleen or bone-marrow. The last one which was sacrificed and examined was proved infected culturally, six months after inoculation. No conclusion therefore can be drawn from this result.

It is difficult to believe that the change in the food of the flies from blood to fructose would *per se* cause an increase in the virulence of parasites, and the following explanation of the sequence of events leading to the results obtained in this series of transmission experiments is tentatively offered: In previous years when sandflies were fed on blood only, with each successive meal not only was the multiplication and development of the flagellates retarded to a certain extent, for which reason no 'blocking' was detected, but the infectivity of the flagellates in the flies was also adversely affected; the animals bitten by such flies therefore were inoculated with small doses of relatively avirulent parasites with the result that very few developed infections. When on this occasion flies were fed on raisins the multiplication and development of the flagellates proceeded uninterruptedly, and the infectivity of the flagellates, which developed from L. D. bodies of average virulence, such as would seem the patients used for infecting the flies harboured, was uninfluenced one way or the other. Transmissions were therefore effected as a result of larger doses of parasites of average infectivity.

Whatever the reason for these results, they are exactly what were expected of the sandfly *P. argentipes* since evidence connecting this species with the spread of kala-azar was first discovered, and it is felt that the final test to incriminate the sandfly as the vector of kala-azar by transmission to a human being can now be undertaken with greater confidence of success.

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EXPERIMENTAL INTESTINAL MYIASIS.

BY

C. STRICKLAND, M.D.,

AND

D. N. ROY, M.D., D.T.M.

(From the School of Tropical Medicine, Calcutta.)

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THE experimental work on the survival of muscoid larvæ during their passage through the alimentary canal of cats and dogs reported by Causey (1938) has led us to publish a short account of the results of similar experiments undertaken in 1931 and mentioned in that year's *Annual Report of the Calcutta School of Tropical Medicine.*

EXPERIMENTAL.

Experiment 1.—A healthy-looking rabbit was forcibly fed with a very large number of first-stage larvæ of *Musca vicina* in milk. Daily examinations of its stools did not show the presence of any larvæ, and the animal's health was not impaired.

Experiment 2.—A very large number of first- and second-stage larvæ of *Sarcophaga ruficornis* was given to a dog with its morning's food. That evening it refused its meal and passed three liquid motions. On the next day hæmorrhagic stools were passed. and it again refused all food. Recovery occurred after about a week but at no stage of the illness was any larva found in the stool.

Experiment 3.—A very large number of first- and second-stage larvæ of *Chrysomyia megacephala* were introduced into the stomach of a pup with milk. From the next day it began to pass thin watery stools mixed with blood. Considerable wasting was noticed and the pup died on the ninth day. Post-mortem examination showed that the stomach was considerably distended with gas and contained three intact adult flies (*C. megacephala*) floating in a watery mucoid substance. No empty pupal case or any larva was found either in the stomach or in the intestine.

Experiment 4.—The presence of eggs of *Chrysomyia megacephala*, when given to a rabbit and also to a dog three hours after the eggs were laid, could not be detected in their fæces.

The ætiology of intestinal myiasis is therefore a moot point.

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THE HELMINTH PARASITES OF DOGS IN CALCUTTA AND THEIR BEARING ON HUMAN PARASITOLOGY.

BY

P. A. MAPLESTONE, D.S.O., D.SC., M.B., B.S., D.T.M.,

AND

N. V. BHADURI, M.B., B.SC. (Cal.).

(From the Helminthological Inquiry under the Indian Research Fund Association, School of Tropical Medicine, Calcutta.)

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It is important from the public health point of view to know what parasites one is likely to find in domestic animals in various localities because certain of them are transmissible to man. A complete world check-list of all the worms recorded from any species of animal is very valuable, but it is not of much practical use for a single locality because many of the parasites in the list will not be found there and so a number of prophylactic measures that would be indicated from the full list will probably be unnecessary.

The publications of Southwell (1930), Bhalerao (1935) and Baylis (1936, 1939) have supplied a full account of existing knowledge of helminth parasites in India, but India is a big country with many varieties of climate so the parasitic fauna of animals probably show considerable variation in different places, accordingly the above records are too comprehensive for local application and are therefore, to some extent, open to the same objection as a world check-list.

The two animals of greatest importance to man are the cat and the dog on account of their close association with him. Chandler (1925) made a survey of the worm parasites of cats in Calcutta, but as far as we are aware the only reported survey of dogs in India was a list without any comment published by Acharya (1933) in which he recorded seven different species of helminths in 49 dogs in Lucknow. Four of the same worms (*Ancylostoma caninum*, *Dipylidium caninum*, *Tænia hydatigena* and *Toxacara canis*) were found by us, whereas we failed to find *Tænia pisiformis, T. multiceps* and *Spirocerca lupi.*

On two occasions we have examined 100 stray dogs captured in the Calcutta streets, to gain some idea of the helminth parasites they harboured. On the first occasion, some years ago, the main object of the survey was to ascertain the frequency of *Echinococcus granulosus*, and on the second occasion (1938-1939) the special subject of investigation was to find if *Tænia ovis* was present. Both times all the organs (alimentary canal, liver, gall bladder, kidneys, lungs, heart, pleural, peritoneal and pericardial cavities) were carefully scrutinized and all the worms collected and identified. In the second series of dogs a search for *Trichinella spiralis* was also made by mincing the diaphragms, digesting them overnight with pepsin and hydrochloric acid and extracting them in a Baermann apparatus. The result is negative so it is not referred to again nor is it to be found in the list, because this worm has never been recorded from the dog in India. The blood was also examined for microfilariæ.

The check-list below includes all the worm parasites found in dogs in India of which an authentic record or records exist. Our positive findings are shown by a figure opposite the name of the worm in each series, and as 100 dogs were examined each time they may be read as percentage incidence. Worms previously recorded but not found by us are indicated by a dash opposite the names.

LIST OF PARASITES.

	Series 1.	Series 2.
Trematodes.		
Alaria alata	—	11*
Echinochasmus perfoliatus	14	39
Echinochasmus sp.	—	3*
Heterophyes heterophyes	13	72
Opisthorchis felineus	—	8
,, *noverca* var. *lobata*	—	—
,, ,, ,, *orbiculata*	—	—
Paramphistomum sp.	—	2*
Paryphostomum sufrartyfex	1*	—
Platynosoma sp.	—	1*
Pseudamidostomum truncatum	—	—
Troglotrematidæ (new genus ?)	—	1*

* Species not before recorded from dogs in India.

LIST OF PARASITES—*contd.*

	Series 1.	Series 2.
Cestodes.		
Anoplocephala sp.	—	—
Diphyllobothrium mansoni ?	1*	—
,, *ranarum*	—	—
,, *reptans*	—	—
Dipylidium caninum	42	79
,, *sexcoronatum*	—	—
,, sp.	—	—
Echinococcus granulosus	2	18
Hymenolepis sp.	—	—
Mesocestoides lineatus	—	—
,, *tenuis*	—	—
Tænia gaigeri	—	4
,, *hydatigena*	5	29
,, *multiceps*	—	—
,, *ovis*	—	—
,, *pisiformis*	—	—
,, *serialis*	—	—
,, sp.	—	—
Nematodes.		
Ancylostoma braziliense	53	69
,, *caninum*	99	91
,, *duodenale ?*	—	—
Dioctophyme renale	—	—
Dipetalonema reconditum	—	—
Dirofilaria immitis	—	—
,, *repens*	—	—

* Species not before recorded from dogs in India.

LIST OF PARASITES—*concld.*

	Series 1.	Series 2.
Nematodes—concld.		
Dracunculus medinensis	—	—
Gnathostoma spinigerum	—	8
Microfilaria lewisi	..	—
Oslerus osleri	—	—
Rictularia cahirensis	..	1*
Spirocerca lupi	--	--
Thelazia callipæda	--	—
Toxascaris leonina	—	--
Toxocara canis	82	87
Trichostrongylus colubriformis	—	6*
Uncinaria stenocephala	--	--
Acanthocephala.		
Echinorhynchus sp.	—	1*

* Species not before recorded from dogs in India.

TREMATODES.

Alaria alata (Goeze, 1782) has been recorded from carnivora in Europe, but it does not appear to have been recorded in India before. There is no evidence it has ever occurred in man.

Echinochasmus perfoliatus (von Ratz, 1908) which is found in dogs in Europe and in many parts of the Far East appears to be a fairly common dog parasite in Calcutta. It has been found in man in Japan but nowhere else so is only a rare human parasite. It is acquired by eating uncooked fish in which the metacercariæ encyst.

Heterophyes heterophyes (von Siebold, 1852) was first found in man in Cairo and it is now known to be common in the Nile valley. It has since then been found in many countries in Asia and is now known as a frequent parasite of cats, dogs, foxes, etc. as well as of man. Our figures for both series indicate that it is a very common parasite of dogs in Calcutta, but strangely Chandler (1925) failed to find it in 250 cats in the same locality. The only record of its probable presence in man in India was the finding of eggs that were identified as those of *H. heterophyes* in the stools of two patients at the Calcutta School of Tropical Medicine

18 or 19 years ago, shortly after it was opened, and neither the eggs nor adult worms have been seen in the many thousands of stools that have been examined there since. It must accordingly be classed as an extremely rare parasite of man in this country. Like *E. perfoliatus* the infective larval stage encysts in a fish.

Opisthorchis felineus (Revolta, 1884) has been found in numerous species of carnivora in Europe and Asia and is common in man in parts of East Prussia and Siberia. It is also found in man in Japan and French Indo-China, and although some publications include India as a country where it is found in man we are inclined to agree with Chandler who said it was not found in man here. It appears to be much commoner in cats than in dogs as Chandler found it in 61 per cent of the animals he examined. This fluke is also dependent on the eating of raw fish for its propagation.

Paramphistomum sp.—All the specimens recovered are immature so it is impossible to identify the species, but it appears to be near *P. explanatum.* As far as we can ascertain no worm of this group has ever been found in dogs before. It is probably of no importance as a parasite of man.

Paryphostomum sufrartyfex (Lane, 1915) Bhalerao, 1931.—This parasite was first described by Lane (1915) from a girl in Assam, being passed after an anthelminthic. As far as we know the only other time it has been recorded in a human being is when the eggs of this species were found in the stool of a girl, also from Assam, for two days after admission to our hospital. The eggs then disappeared and daily examination for over a week failed to reveal any more eggs or adult worms. Leiper (1911) described a closely similar fluke from man in the Malay States. He gave it the name *Echinostoma malayanum* which has now been changed to *Paryphostomum malayanum* by Bhalerao. Leiper expressed the opinion that it might be a normal parasite of dogs and cats, and Lane, on account of the apparent rarity of his species as a human parasite, said it probably had a normal host other than man. Lane's opinion of the rarity of *P. sufrartyfex* in man is confirmed by our later experience and his opinion of another animal being the normal host is also confirmed by the fact that, according to Bhalerao (1931), Maplestone (1930*a*) found it in 33 per cent of the pigs he examined in Calcutta. As far as we know this genus has not been found in dogs before and this is of considerable interest in view of Leiper's remark that his closely allied Malayan species might be found in the dog.

Platynosoma sp.—Only a single specimen was found so it is not possible to define the species. The genus is much commoner in birds than in mammals, but one species has been found in cats in America and in the Malay States. As far as we know it has never been found in the dog before.

Troglotrematidæ sp. Genus ?—This family which contains the genus *Paragonimus* (species *P. westermanii*) is a fairly common and important parasite of man in many parts of the Far East, and has been recorded from the tiger in India. This worm will be described and named by Dr. Bhalerao to whom material has been sent.

CESTODES.

Diphyllobothrium mansoni (Cobbold, 1882).—The single specimen that we have recovered belonging to this genus is possibly *D. mansoni* which has not been found in India previously. It is common in dogs, cats and wild carnivora in China, according to Mönnig (1934), and the larval stage (*Sparganum*) occurs in reptiles, birds and many mammals including man. Our tentative diagnosis is based on the number and distribution of the testes in mature segments, which is stated to be 380 to 540, and two of the segments in our specimen contained about 540 and 570, respectively. Joyeux and Baer (1927) say that the number and arrangement of the testes is the only reliable difference between *D. mansoni*, *D. reptans* (Diesing, 1850) and *D. ranarum* (Gastaldi, 1854) in which two species the testes are limited to the lateral fields and number 144 to 220 and 100 to 110, respectively.

Joyeux and Houdemer (1928) say that *D. mansoni* and *D. felis* (Creplin, 1825) are very much alike. Chandler (1925) recorded *D. decipiens* (Diesing, 1850) from the domestic cat in Calcutta and Southwell (1928) recorded *D. felis* from several kinds of *felidæ* in the Calcutta Zoological Gardens. Southwell (1930) gives *D. decipiens* as a synonym of *D. felis*. The testes of *D. felis* do not appear to have been enumerated but they are described as ' numerous ' by Southwell, and comparison of our specimen with Chandler's drawing of *D. decipiens* suggests that we are dealing with the same species. It will be seen from this discussion that there is considerable confusion regarding the differentiation of several of the species of *Diphyllobothrium* so we do not feel justified in making a definite specific diagnosis of our specimen. It also adds support to the statement of more than one authority, that the diagnostic characters of some of the species are so variable and indefinite that there is considerable probability that several of these so-called distinct species are identical, and it raises the question whether *D. mansoni* should not also be considered a synonym of *D. felis*.

Dipylidium caninum (Linnæus, 1758).—According to our figures this worm appears to be the commonest cestode in dogs in Calcutta, and other records show that it is very widely distributed in India. In spite of its frequency it has never been found in man in India although the literature contains numerous references to it as a parasite of human beings in other parts of the world. When found it is nearly always in children, and as it can apparently only be acquired by swallowing fleas containing the cysticercoids it is never likely to become a common parasite of man no matter how common it may be in dogs. Chandler also found it in 43 per cent of cats.

Echinococcus granulosus (Batsch, 1786).—The figures from our two series of dogs show considerable difference, being two in the first series and 18 in the second. This is not considered to be of any significance as evidence that the infection is increasing, because the stray dog population of Calcutta is very large and the difference in our two series is probably only an error caused by taking too small samples. Nevertheless it indicates that this infection is constantly present and it represents a real danger to the people of Calcutta. We consider that this may be taken as still further indirect evidence of which we have collected a good deal

in recent years, that hydatid disease is commoner in India than the available records show.

Tænia gaigeri (Hall, 1916).—This worm was first described by Hall (1910) from material sent to him by Gaiger from Lahore, and this appears to be the only previous record of the adult. Although only four dogs harboured this species the number of worms were fairly large so that ample material was available for study. This has been examined in detail and several variations from Hall's description have been noted. An account of these will be published elsewhere in the hope of forestalling possible subsequent multiplication of species that may occur if they are noted by later workers with insufficient material on which to base their value as specific characters. It is probable that something of this kind has taken place in the case of several other of the cestodes of dogs because there is a great deal of doubt if all the species are really valid.

In the case of *Tænia multiceps, Tænia gaigeri* and *Tænia serialis* we have followed Southwell (1930) who removed these species from the genus *Multiceps* in which they had been placed by Hall (*loc. cit.*). Hall's reason was based on the fact that these species have a *Cœnurus* for their larval stage. Southwell rightly points out that the anatomy of these three species of *Tænia* is almost identical with others found in dogs and as the only way of ascertaining what form of larva gives rise to a given adult is by feeding experiments it is unsound in this group to use larval characters for generic diagnosis.

Tænia hydatigena Pallas, 1766.—This worm is common in Calcutta dogs; its presence calls for no special comment.

Tænia ovis (Cobbold, 1869) Ransom, 1911.—The adult is stated to have been found in Lahore but the larva has never yet been recorded in India. On account of its complete absence from all the dogs of both our series it is probably a very uncommon parasite of the dog in India and is therefore unlikely to be a source of human cysticercosis, as was suggested by us previously. Our present conclusion is supported by the fact that we have been unable to find any *Cysticercus* in mutton, either at the Calcutta slaughter-house or in butcher's shops, in periodical inspections of meat extending over several years. Some butchers say that they occasionally see bodies in mutton which may well be *Cysticercus ovis* from their description but they have never been able to produce any for our inspection although we have a standing offer of a reward for such material.

Nematodes.

Ancylostoma braziliense Gomez de Faria, 1910.—This is a common parasite of cats and dogs in many parts of the world, as well as having been found in numerous wild carnivora. It is also an occasional parasite of man and in this respect it is of special interest in India as most of the records of its occurrence in man have been made here. It was first seen in man by Lane (1913) in gaol prisoners in Bengal where he found 9·3 per cent of 150 men harbouring a few *A. braziliense*, along with other hookworms. The next published record is that of

Chandler (1927) in reference to a few cases of this infection with this worm in Burma, some reported by Jolly and later ones by himself. Chandler failed to find this species in a large number of worms passed by man in Calcutta and concluded that for some unknown reason it failed to infect man in this city. Since then we have often found it in man in Calcutta, and in five years we have recorded it in our annual reports in a total of 22 out of 943 (2·3 per cent) of our hookworm cases. These were all hospital in-patients in whom all the hookworms passed are identified. We have never found a pure *A. braziliense* infection in man and never more than three or four worms of this species in one case.

In several other parts of the world this species is of particular importance as it produces a severe and often long-standing skin condition known as 'creeping eruption'. For some unexplained reason this condition is very rare in India and Maplestone (1933) investigated the matter experimentally when he produced only a mild and abortive skin eruption, which cleared up spontaneously, with the larvæ of this species, and he found *Necator americanus* more active in this respect. One or two of the experimental cases acquired a mild and transient infection with *A. braziliense* all of which disappeared in a few days without treatment. This fact suggests that gut infection may be often acquired with *A. braziliense* but is not often found, as it lasts such a short time.

Ancylostoma caninum (Ercolani, 1859) von Linstow, 1889.—This species has also been found in many wild carnivora as well as in dogs, but Chandler failed to find it in cats. The only record of this worm in man is that of Manalang (1925) in the Philippines so it is such a rare parasite of man that its high incidence in dogs in Calcutta is of no real importance, and it did not appear active in producing creeping eruption, according to Maplestone's experiments.

Gnathostoma spinigerum Owen, 1836.—From the summary by Baylis (1939), it appears that the only previous record of this worm in the dog in India is a collection of specimens in the British Museum from a dog in Burma (Rangoon), but Chandler found it very common in cats. The eight dogs in which we found the worms had the typical large fibrotic tumours in the stomach wall with an opening into the stomach from the central cavity of the tumour in which the adult worms live, a condition that has been described in several felidæ, including domestic cats, so it is evident that dogs are also favourable hosts for the adult stage of these worms. Immature gnathostomes have been reported in a good many human beings in whom they burrow energetically through any tissue they may encounter in their migrations, except perhaps bone. If vital organs are involved in these burrowings, urgent symptoms may arise. It is now known that the infective larval stage is encysted in several species of fresh-water fish so that the countries where this parasite is common in animals and in which the inhabitants eat raw fish are those in which it is most frequent in man, thus resembling certain flukes. Although raw fish is hardly ever eaten in India five cases have been recorded (Maplestone, 1929, 1930*b*, 1931; Maplestone and Bhaduri, 1937; Maplestone and Sundar Rao, 1939). As all these cases were found by one worker and they came to Calcutta from various parts of Bengal, it is possible the condition is commoner in this country than emerges from these few records.

Rictularia cahirensis Jagerskiöld, 1904.—A single specimen of this worm was found. It has been recorded twice before in India from a civet cat by Baylis and Daubney (1923) and by Srivastava (1937).

Trichostrongylus colubriformis (Giles, 1829) Ransom, 1911.—Herbivorous animals are the normal host of this worm, in which it has been found in many parts of the world, including India. Baylis (1936) also names species of monkeys and man as hosts of this worm and he says Boulenger in 1920 reported *Trichostrongylus* eggs in a small proportion of Indian hospital patients in Mesopotamia, and that Sweet estimates that 0·4 per cent of the population in Mysore is infected with it. In addition to these records we have been publishing, in our annual reports since 1930, the incidence of all intestinal helminth eggs found in our hospital and out-patient department, and we have found *Trichostrongylus* eggs 156 times in 14,446 examinations (1·07 per cent), but we have never been able to recover the adult worms. This is the first time this worm has ever been found in a dog, and the fact that it was present six times in the second series of 100 dogs indicates that it is more than a chance occurrence. Apart from the academic interest it is probably of some practical importance also, regarding the source of man's infection, because dogs are as a rule in much closer contact with man than are his sheep and cattle, and so are a more probable source of his infection.

Toxocara canis (Werner, 1782).—This is a common and cosmopolitan parasite of dogs, so its high incidence in Calcutta calls for no special comment, and as it has been only once recorded from man (in Egypt) its chance of becoming a frequent parasite of man seems negligible.

Acanthocephala.

Echinorhynchus sp. ?—A single immature specimen was obtained, which was impossible to identify more accurately on account of its lack of development. According to Baylis (1929) *Echinorhynchus canis* Railliet, 1893, is probably *Moniliformis moniliformis*, and he considers that it is doubtful if '*Echinorhynchus canis*' Porta, 1914 is the same species, as has been suggested by others, because, like our single specimen, it is not of sufficient maturity.

Summary.

1. Twenty-one different helminth parasites have been recovered from the 200 dogs examined by us. The complete list of worms found in dogs in India includes 50 species and of these ten are now recorded by us in this paper for the first time.

2. One of them *T. colubriformis*, which is a common parasite of herbivora and occasionally of man, has never before been found in a carnivore.

3. No member of the genus *Paramphistomum* or of the family *Troglotrematidæ* has ever before been recorded in dogs, and *P. sufrartyfex*, whose known distribution is limited to North-Eastern India, has hitherto been found in pigs several times and in man twice.

4. The two flukes *H. heterophyes* and *O. felineus* are common parasites of man in other parts of the world, so they are important potential parasites of man in India. As both these worms depend on eating of raw fish for their transmission, the Indian is protected from acquiring them by the fact that he rarely indulges in this form of diet. The same set of circumstances also limits the incidence of *G. spinigerum* infection in human beings in India.

5. By far the most important parasite of the dog in Calcutta as a menace to the health of man is *E. granulosus* the incidence of which is fairly high for city dogs.

6. The absence of *T. ovis* from all our dogs, coupled with our failure to identify *C. ovis* in mutton sold in Calcutta, disposes of our previously held opinion that this tapeworm was a possible source of at least some of the cases of human cysticercosis, of which an unduly large number has been seen in British troops in India during the past few years.

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STERNAL PUNCTURE IN FILARIASIS.

BY

L. EVERARD NAPIER, F.R.C.P. (Lond.),

C. R. DAS GUPTA, M.B. (Cal.), D.T.M.,

AND

S. SUNDAR RAO, L.M.P.

(From the School of Tropical Medicine, Calcutta.)

[Received for publication, May 20, 1940.]

STERNAL-PUNCTURE studies on filarial patients were carried out both during the day and night with two objects in view: firstly, to see if they would throw any light on the mechanism of nocturnal periodicity of the filarial embryo in the blood, and, secondly, to ascertain if there was any evidence of cytological changes in the marrow in these patients.

Fifty-three cases were investigated by this method. All the patients either showed microfilariæ in the blood during the night or were clinically typical cases of filariasis. The actual findings were as follows :—

Showing microfilariæ in the blood at night	25
Did not show microfilariæ in the blood at night ..	13
Night blood was not examined (out-door cases) ..	15

Microfilaria counts of the peripheral blood, venous blood and of blood drawn by sternal puncture were made in these patients, both during the daytime and at night. In a few cases, examination of fresh blood from the finger, vein and sternum was made to see if the embryos showed any difference in their activity; none was noted. All of the patients examined had some type of filarial lesion, such as lymphangitis, elephantiasis, or chyluria. The patients were representative of all races and ages, and both sexes. The sternal puncture was done by the usual method with a Salah needle. About 0·5 c.c. of fluid was taken; this was well mixed in the syringe, thick and thin films were made, and the balance was put

into a tube with oxalate powder for the total nucleated-cell count. The results of these studies are summarized below :—

Incidence of microfilariæ in sternal-puncture fluid.—In 41 sternal punctures which were done during the day only, no microfilariæ were found. In the remaining 12 cases the findings were as given in Table I :—

TABLE I.

Serial number.	Sternal-puncture fluid; microfilariæ count in 0·2 c.c. of blood.		Peripheral blood: microfilariæ count in 0·2 c.c. of blood.
	Day.	Night.	Night.
4	0	5	50
5	..	3	39
6	..	98	64
7	..	13	140
12	1	..	129
17	1	5	40
24	1	..	60
48	0	5	60
49	..	2	9
51	..	3	8
52	..	1	2
53	..	1	8

Out of 53 cases, microfilariæ were found in the sternal fluid in 12 cases, in three during the day and in ten during the night (one both day and night).

Of 46 sternal punctures done during the day, three showed microfilariæ in the sternal-puncture fluid; all these three were in the hospital and showed a very heavy infection in the peripheral blood at night. Of the ten cases done at night all showed microfilariæ.

Of the three cases in which sternal puncture was done both during day and night, one showed microfilariæ on both occasions and the other two at night only.

In all the cases examined a much larger number was found in the peripheral blood than in the sternal-puncture material.

In two cases in which a note was made of the numbers of microfilariæ in the capillary and venous blood and in the sternal-puncture fluid, there were more microfilariæ in the capillary blood, and least in the sternal-puncture fluid.

Discussion.

Although in heavily infected cases microfilariæ may be detected in the peripheral blood even during the day, all the cases examined showed them only during the night. In general, whenever the microfilaria count of the peripheral blood was high, there was a correspondingly large number of microfilariæ in the blood drawn from the vein or by sternal puncture, whereas cases which showed only slight incidence of microfilaria in the peripheral blood showed fewer microfilaria in the venous blood or in the blood drawn by sternal puncture. The microfilaria count in the peripheral blood was generally higher than that in the venous blood which again was much higher than that in the sternal-puncture blood. The sternal-puncture blood taken at night always showed a higher microfilaria count than the same blood drawn during the day. There was no evidence to show that filarial embryos sheltered in the bone-marrow during the day, nor was there any sign of their destruction going on in the bone-marrow.

Bone-marrow cytology.—These studies do not indicate that there was any anæmia associated with filarial infection, nor that there was any change in the normal proportions of the various cells in the sternal-puncture material; a summary of the cytological findings is given in Table II :—

Table II.

Result of cytological examination of bone-marrow.

	Number.	Range.		Mean.	Standard deviation of mean.
		Lowest.	Highest.		
Total nucleated cells, per c.mm. ..	31	20,000	309,000	111,6780	± 64,8315
Red cell series, per cent of nucleated cells—	53	10	70	27·6058	± 11·7458
Megaloblast	53	0	3·25	0·9170	± 0·7366
Erythroblast	53	0	3·3	0·4924	± 0·6619
Macroblast	53	0	8·75	2·0750	± 1·7675
Normoblast	53	7·5	63·2	24·1718	± 10·4505

TABLE II—*concld.*

	Number.	Range.		Mean.	Standard deviation of mean.
		Lowest.	Highest.		
White cell series—					
(A) Granular series	53	21·8	81·8	54·5285	± 11·4210
Myeloblast	53	0	0·75	0·1500	± 0·2670
Premyelocyte	53	0	2·0	0·4358	± 0·4409
Neutrophil myelocyte ..	53	1·0	18·75	8·2991	± 3·2061
Neutrophil, young forms ..	53	0·40	8·25	2·7566	± 1·7257
Neutrophil, band ..	53	12·4	60·0	33·9075	± 9·1938
Neutrophil, segmented ..	53	0	23·6	5·3368	± 5·0232
Eosinophil myelocyte ..	53	0	4·0	1·4453	± 0·9498
Mature { Eosinophil ..	53	0	7·4	2·7687	± 1·6539
Mature { Basophil ..	53	0	1·2	0·0821	± 0·2090
(B) Non-granular series ..	53	4·3	35·5	17·8888	± 6·7963
Lymphocyte	53	2·9	34·5	15·6764	± 7·2623
Large mononuclear ..	53	0	3·5	1·5846	± 0·9386

SUMMARY.

1. Sternal puncture was done in 53 cases of filariasis. In three out of 46 cases done during the daytime and in ten out of ten done at night, microfilariæ were found. In all instances there were more microfilariæ in the peripheral blood than in the sternal-puncture fluid.

2. There is thus no evidence that microfilariæ shelter in the bone-marrow during the day, or that they are destroyed in the marrow.

3. The cytological analysis of the material does not suggest that there is any change in the bone-marrow in this infection, and in fact the nucleated-cell counts might well be taken as a normal series.

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STUDY OF FILARIAL INFECTION IN RATANPUR (CENTRAL PROVINCES).

BY

S. SUNDAR RAO.

(Filariasis Inquiry under the Indian Research Fund Association, School of Tropical Medicine, Calcutta.)

[Received for publication, May 3, 1940.]

IT is well known that two species of filarial parasites are prevalent in India, viz. *Wuchereria bancrofti* and *Wuchereria malayi*. The former infection is transmitted chiefly by the *Culex fatigans* and the latter by *Mansonioides annulifera*. *W. bancrofti* is observed only in urban localities, while the *W. malayi* is confined to the rural parts. In some localities both the infections are prevalent. The infection caused by *W. malayi* is characterized by elephantiasis of the extremities, while the infection due to *W. bancrofti* causes chyluria, lymphvarix, chylocele, hydrocele and elephantiasis of the genitals in addition. The infection can be easily diagnosed by an examination of the embryos which are present in the blood of infected population in the endemic areas. *Culex fatigans* is an urban mosquito and breeds in collection of water containing organic matter. *Mansonioides* breeds in ponds in rural areas and lays its eggs on the leaves of pistia plant which grows wild in these ponds. 'Species sanitation' is important for prevention of infection. Breeding of *Culex fatigans* is controlled by periodical treatment of breeding places with larvicides, while the control of breeding of *Mansonioides* is done by clearing away the pistia from the ponds. Thus, in a survey and study of filarial infection in an area particularly with a view to apply preventive measures for controlling it, investigation has to be directed towards finding out the species of the parasite and the carrier which spreads the infection.

Surveys made in the various provinces of India has indicated that *W. bancrofti* infection is much more widespread than *W. malayi*. *W. bancrofti* is mainly observed in towns all over the coastal and water-logged areas in India. Places like Shertalai in North Travancore, Balasore and Patnagarh in Orissa, Comilla in East Bengal and Sylhet in Assam, have been found to be infected only with *W. malayi*. Places

where both the types of infection are prevalent are also commonly met with, e.g. Alleppey (Travancore). It would, therefore, be important to study in detail the actual type of infection prevalent in one locality.

Recently, the author's attention was drawn by Major V. Srinivasan, F.R.C.S., I.M.S., Civil Surgeon, Bilaspur (Central Provinces), to the prevalence of elephantiasis in a village in Bilaspur (C. P.) where the neighbouring localities remained practically uninfected and it was felt that it would be interesting to study the cause of the infection in this locality. This study gives the results of the survey carried out in Bilaspur district and particularly the infected village, viz. Ratanpur.

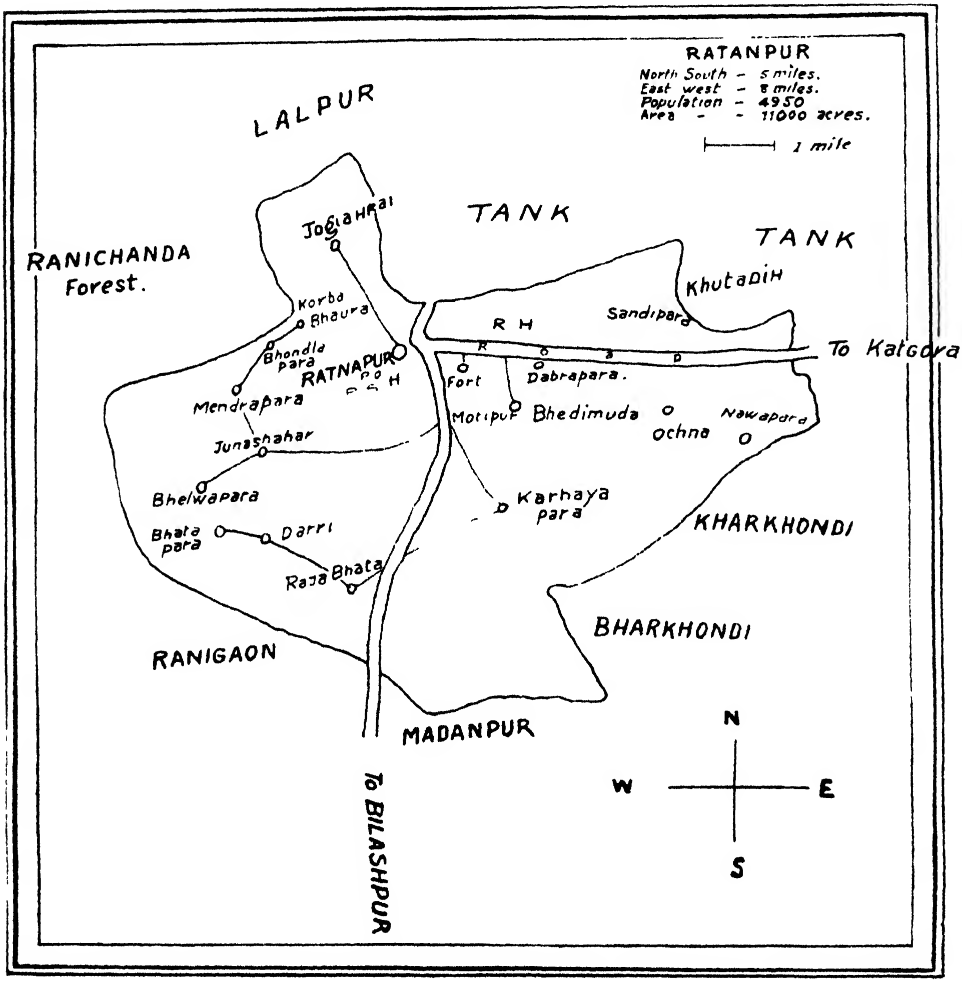
Ratanpur is a town in the Bilaspur Taluk (C. P.) and was the capital of Chhattisgarh for many centuries. It is situated in 22° 17′N. and 82° 11′ E., 16 miles north of Bilaspur by road. The elevation of the locality is 993 feet above the sea-level. The area of Ratanpur proper is about 11,000 acres and has a population of 4,950. The locality is more rural than urban. The population comprises mainly Brahmins, Banias, Karis, Sonars (Hindus). The country around is very uneven with hill ranges rising in various directions. The village is built at the base of Kanda range and lies in a hollow almost surrounded by isolated hills so that nothing is seen of it until one actually enters its precincts. The soil is fertile, being for the most part light and sandy. Agriculture is the chief occupation. The climate resembles closely that of Bilaspur. The temperature in May rises to nearly 116°F. with a range from 82°F. to 107°F. In July the range of temperature is much smaller, being from 75°F. to 87°F. on the average, while in January average maximum and minimum are 82°F. and 55°F. Average annual rainfall is about 48 inches for the district and is received chiefly during the south-west monsoon from June to September. There are many large tanks in the town. Water-supply is obtained from wells and tanks. There are no drains in the town nor conservancy except hand removal of night-soil from a few houses. The majority of the population use scrub jungle or open ground for defæcation. The prevailing diseases in the order of frequency are malaria, filariasis, syphilis, skin disease, dysentery, leprosy, stone in bladder and goitre. Ratanpur is divided into a number of wards, the biggest ward being Karhayapara and this is inhabited mostly by Brahmins. The Map shows the different wards of Ratanpur.

Filarial infection in Ratanpur.

Ratanpur has long been known to be infected with filariasis. A survey of the town carried out by the author early this year (March 1940) showed that the filarial infection in this village is exclusively due to *W. malayi* and the filarial diseases found in this area are characteristic of this infection, viz. elephantiasis of the legs and hands, lymphangitis and abscess. No case of hydrocele, chyluria, lymphvarix or elephantiasis of genitals was observed in this area. A house-to-house survey showed that out of a population of 2,000 examined 78 had elephantiasis of the legs or hands.

The incidence of filarial infection of Ratanpur was determined by an examination of peripheral blood of the population taken at night between 9 p.m. and 10 p.m. A total of 191 persons representing all the wards of Ratanpur were examined. Thirty-one of them showed microfilariæ in blood (microfilaria rate :

MAP.



16·23 per cent). This may be considered as the gross rate of infection for the town. The infection was found to be exclusively due to *W. malayi*. There was no *W. bancrofti* infection in any of the specimens examined.

Table I shows the results of clinical examination for filarial diseases from a house-to-house survey made in Karhayapara :—

TABLE I.

Showing the types and incidence of filarial diseases in Karhayapara. Total population examined 2,000.

Filarial diseases.		Male.	Female.	Total.	Percentage.
Elephantiasis	one leg	27	8	35	1·75
,,	both legs ..	21	21	42	2·10
,,	fore-arm and hand ..	1	0	1	0·05
,,	fore-arm and leg ..	0	0	0	0
	TOTAL ..	49	29	78	3·90

Elephantiasis of the legs forms the chief type of filarial disease met with in Ratanpur. History of lymphangitis of the extremities recurring periodically is elicited in every case of lymphatic obstruction. Abscesses along the main lymphatic regions or on elephantoid limbs are fairly common. Other manifestations of filarial infection, such as elephantiasis of the genitals, hydrocele or chyluria, do not occur in Ratanpur. This is in agreement with the reports by Brug (1927), Korke (1929), Iyengar (1932) and Sundar Rao (1936) in areas where *W. malayi* occurs.

MOSQUITO SURVEY.

The following species of mosquitoes were found to be prevalent in Ratanpur during the period of survey. The identification was kindly done by Mr. M. O. T. Iyengar, B.A., F.Z.S., Officer-in-Charge, Bengal Malaria Research Laboratory, Public Health Department, Calcutta :—

Mansonia (*Mansonioides*) *annulifera*, *M. uniformis*, *Culex vishnui*, *C. fatigans*, *Anopheles pallidus* and *A. annularis*.

Mansonioides species were found to be breeding in most of the big tanks. All the big tanks were covered with pistia. The breeding of *Culex fatigans* was found to be restricted to small collections of water in broken pots near the wells.

INTERMEDIATE HOST.

Mosquitoes collected from dwelling houses from different parts of Ratanpur were dissected and examined for filarial infection to determine the intermediate host. Out of 90 specimens of different species examined natural infection was found in only two species : *Mansonia annulifera* and *M. uniformis*. The most

common species in the area was *Mansonia annulifera*. Judging from the high prevalence and also from the infection observed under natural conditions it is evident that this species is the chief carrier of filarial infection in Ratanpur. *M. uniformis* also plays an appreciable part in the transmission of the infection. The other species are probably unimportant from the point of view of transmission of *W. malayi* infection in Ratanpur.

Summary.

Ratanpur in Bilaspur district (C. P.), a small rural type of town, has endemic filariasis, the infection consisting entirely of *W. malayi* Brug. The microfilaria rate is 16·23 per cent and the filarial disease rate is 3·9 per cent. Elephantiasis of the limbs is the most common filarial disease. Elephantiasis of the genitals, hydrocele, chylocele and chyluria are entirely absent. The mosquito survey showed a prevalence of *Mansonioides* species. *Mansonioides annulifera* is the commonest species of mosquito in Ratanpur and appears to be the chief transmitter of filarial infection. The breeding places of *Mansonioides* are the large tanks densely covered with pistia.

Acknowledgments.

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PRESERVATION *IN VITRO* OF *MICROFILARIA BANCROFTI* AND A STUDY OF THE MECHANISM OF EX-SHEATHING.

BY

T. BHASKARA MENON, M.D., D.SC., F.R.C.P.,

AND

B. RAMAMURTI, M.B., B.S.

(*Financed by the Indian Research Fund Association.*)

(*From the Department of Pathology, Andhra Medical College, Vizagapatam.*)

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INTRODUCTION.

THE viability of *Microfilaria bancrofti* outside the human body has been generally regarded as very short. It is viable usually for about twelve hours when kept in citrated blood at body temperature and about three days at laboratory temperature (Bahr, 1912). Longer survival periods have been reported ; a week by Bahr *et al.* (1933), thirty days by Coutelen (1929) and four to six weeks by Rao (1933). Joyeux and Sautet (1937) claim that in artificial ' cultures ' the embryo of *Dirofilaria immitis*, the filaria of dog, lives for a period of twelve days and that growth is obtained. Coutelen (*loc. cit.*) also claimed growth with *Mf. bancrofti* and he observed outlines of visceral formations in the embryo in thirty days. Since, in the natural growth of the parasite, the first change observed is the shedding of the sheath of the embryo in the foregut of the mosquito, the mechanism of ' ex-sheathing ' is of interest in any study of growth *in vitro*. A study of the behaviour of *Mf. bancrofti* preserved *in vitro* was undertaken with a view to determine the survival period, growth and ex-sheathing.

MATERIAL AND METHODS.

For keeping the embryos alive the media employed were non-heated Row's medium with and without the addition of glucose, Ringer-Locke's solution

with and without the addition of human plasma, Harley's peptone digest medium, rabbit plasma, hydrocele fluid and 0·85 per cent saline. Current bacteriological technique was adopted with small tubes containing the media. Half c.c. of blood containing on an average five to ten microfilariæ per drop, obtained by vein puncture, was inoculated into tubes containing about 3 c.c. of the media. Whole blood, citrated blood and defibrinated blood were all used. The tubes were kept at room temperature (28°C.) and also at 10°C. Repeated attempts at cultivation were carried out with the various media. For examination of the microfilaria, drops of the media were pipetted off at various intervals.

Various experiments were carried out *in vitro* to determine the mechanism of ex-sheathing. The material used for this purpose was the microfilariæ obtained from recent inoculated media and those that were in the foregut of the mosquito before ex-sheathing had taken place under natural conditions.

Results.

1. *Viability.*—The embryos survived for a few days even in well-made wet-blood preparations. In most of the media they lived up to about a week on an average. In normal saline many lived for about fifty days when maintained at a temperature of about 10°C. This was the longest survival period. In Row's medium the maximum survival period was about three weeks. The free hæmoglobin in the medium did not favour survival. Ringer-Locke's solution also gave similar results. Addition of human plasma to Ringer-Locke or rabbit plasma by itself seemed to be unfavourable. In citrated whole blood kept aseptically the embryos were as active as in blood for a few days, but all of them died about the fourth or fifth day. In all media the embryos survived in diminishing numbers, day by day, after a few weeks. The optimum temperature for the longest viability was found to be about 10°C. Higher temperatures were definitely inimical. A good dilution of the infected blood was noted to prolong survival period. Low temperature as in freezing made the embryos quite inactive, but they regained their activity as the temperature was gradually raised to that of the laboratory temperature. The viability was markedly reduced by septic contamination of the media.

2. *Activity.*—In recent wet-blood films, embryos were so very active that their form could hardly be observed excepting for a quick disturbance in corpuscles around. But in artificial media they lost a great deal of their activity even on the second day. In six weeks the surviving embryos were so very sluggish that it required high magnification to observe movement.

3. *Growth.*—With the embryos of *W. bancrofti* no growth occurred even at the end of fifty days. The microfilariæ remained about 300/8μ throughout the period. The morphology as indicated by the disposition of the granules did not show any change excepting for a slight degenerative haziness on staining in some cases.

Protocol showing the behaviour in various media.

Number.	Medium used.	Temperature, °C.	Viability in days.	Activity.	Growth, change in morphology.
1	Row's medium ..	28–29	7	Moderate for two days, feeble later.	*Nil.*
	do. ..	37	2	..	*Nil.*
	do. ..	10	17	Moderate for two days, feeble later.	*Nil.*
2	Row's with glucose, 0·12 per cent.	10	10	do.	*Nil.*
3	Ringer-Locke ..	10	15	do.	*Nil.*
4	Ringer-Locke, human plasma.	10	6	do.	*Nil.*
5	Hydrocele fluid ..	10	3–5	Marked for two days only.	*Nil.*
6	Rabbit plasma ..	10	4	Moderate	*Nil.*
7	Harley's	10	7	do.	*Nil.*
8	Glucose broth ..	10	7	do.	*Nil.*
9	Citrated whole blood	10	3–4	Good	*Nil.*
10	0·85 per cent saline ..	10	40–50	Feeble	*Nil.*

Ex-sheathing was observed in some cases.

4. *Ex-sheathing.*—In some of the saline tubes it was observed that about the fourth day many embryos had lost their sheaths. Many empty sheaths could be observed in the field along with ex-sheathed embryos. Experiments were therefore undertaken to determine the ex-sheathing factor.

(*a*) *pH variation*.—To determine if any alteration in the pH of the mosquito's stomach juices is the deciding factor, *Mf. bancrofti* were studied in a series of isotonic phosphate buffer solutions ranging from 4·1 to 10 in pH and consisting of about thirteen in series. The embryos used for this purpose were obtained from recently inoculated media. Buffers were prepared with phosphates so that they might not have a toxic effect. The method adopted was to take a drop of the medium on the hollow of a hanging drop-slide, search for a sheathed embryo in it and flood it with the buffer in question. As the pH of the medium itself could be taken as 7, that of the mixture was generally assumed to be very nearly equal to that of the solutions used. Microfilariæ were observed under the microscope. This process was repeated a number of times with each buffer solution. Another method was to incubate the mixtures of culture-buffer solutions mixed in the ratio of about 1 : 10, observations being made at varying intervals.

(*b*) *Artificial digestion with pepsin*.—Low-grade dilutions of pepsin were employed and the method of testing as with buffers was followed.

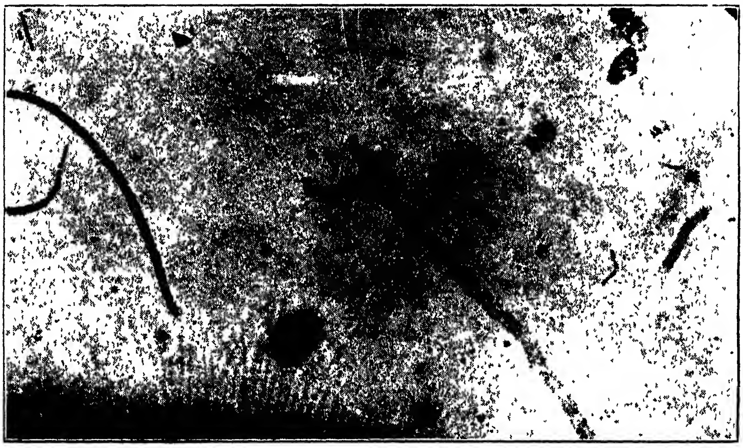
(*c*) *Temperature variation*.—Chilling of wet-blood film preparations and whole blood was studied. Repeated attempts at freezing and then allowing the preparations to thaw at room temperature were made. The technique adopted for this was to place the preparation wrapped up in blotting-paper between two blocks of ice. When the film was beginning to freeze it was taken out and examined under the microscope while thawing was in progress.

(*d*) *Alteration in the viscosity of the medium*.—Though freezing itself alters the viscosity, solutions of fibrin in low dilutions were employed for this purpose. The same method of examination with a hanging drop-slide was followed.

The experiments with buffer solutions, artificial digestion with pepsin, chilling and increase of viscosity did not cause ex-sheathing. The embryo became more active for a time and less viable with acid buffers, while with alkaline buffers their viability was longer, but activity was not marked. Even in 2 per cent bicarbonate solution the embryos lived for about two hours. Ex-sheathing, however, did not occur. Artificial digestion with pepsin while completely hæmolysing the red blood cells in the field, did not affect the sheath of microfilariæ though their viability was much reduced. Temperature variation—chilling and thawing—also failed to cause ex-sheathing. When cooled the microfilariæ became almost immobile, but on thawing, they slowly regained activity. Fibrin also gave negative results.

However, the presence of many ex-sheathed embryos and empty sheaths in recently inoculated media pointed to the existence of some mechanism. In artificial media the formation of small leucocyte-fibrin masses derived from blood could be observed. The microfilariæ seemed to concentrate around and within the semi-solid matrix of these masses in great numbers as if by a process of taxis. They showed a definite tendency to pass through the mass by vigorous wriggling movements. During this process the loose posterior end of the sheath became attached to the mass, the sheath was ruptured and the embryo slowly appeared to wriggle out. The whole process took from a few hours to twenty-four hours before the embryos could be free.

PLATE XV.



Showing an ex-sheathed *Mf. bancrofti* and an empty sheath in artificial medium on the tenth day. × 260.

This mechanism was found to be exactly similar to what took place in observations carried out on the mosquito's stomach full with infective blood feed. The blood clotted and became viscid in the foregut and it could be observed that in about twelve to twenty-four hours after the feed ex-sheathed embryos had wriggled out of the clots and even the stomach wall.

Discussion.

The work of Coutelen (*loc. cit.*) on *Mf. bancrofti* and of Joyeux and Sautet (*loc. cit.*) on *Mf. immitis* suggest change in artificial media in the direction of growth. Coutelen described that the length was increased by a fifth and that the width was doubled in thirty days, and that there appeared digestive, excretory and genital outlines from the active multiplication of genital or somatic cells. Joyeux and Sautet, on the other hand, claimed growth in length even as early as third day, but they thought that this was more a degenerative change. Rao (*loc. cit.*) has not mentioned any morphological change even though the embryos were kept alive for four to six weeks. Our experiments show only mere survival in artificial media for a considerable period (about fifty days), but no growth or developmental changes were observed. The disposition of the granules remained constant, but some appeared more indistinct due to degeneration. The embryos were mostly inactive after the first few days. If one considers the natural life cycle of the parasite the microfilariæ represent a developmental stage from which further differentiation is possible only after ex-sheathing and life in the mosquito's tissues. So far in artificial media a suitable environment has not been obtained and consequently changes in morphology are not in evidence. Concentration methods such as those described by Drinker, Augustine and Leigh (1935) in the filariasis of dogs by hæmolysing the blood with hypotonic saline and centrifugalization have not been successful with *Mf. bancrofti* and the rôle of sepsis in reducing the viability was difficult to overcome.

The demonstration of the long survival period of *Mf. bancrofti* in artificial media would raise the question of their longevity in natural conditions in the human host. But in the absence of a defence mechanism, such as is possible in the human host, no definite conclusions can be drawn. Evidence for the presence of a defence mechanism is suggested by the work of Lane (1933-34) who has demonstrated the presence of dissolution and disintegration of microfilariæ in the reticulo-endothelial network of the lymphatic sinuses. The 'adhesion phenomenon' demonstrated by Pandit, Pandit and Iyer (1929) suggests the presence of cytological factors that might be inimical to the life of the microfilaria in the blood.

The study of the ex-sheathing mechanism is also of great interest. Knott (1935) has reported the occurrence of ex-sheathing *in vivo* of an immune filarial subject after transfusion of blood from a microfilarial carrier. He comes to the conclusion that there is a definite immunity in such subjects since all the injected microfilariæ were very quickly destroyed. The adhesion phenomenon also suggests the existence of an immunity process. The present study raises the question

whether the formation of leucocyte-fibrin masses in artificial media is similar to the adhesion phenomenon in causing ex-sheathing *in vivo* of an immune subject. Whether the ex-sheathing itself is a first step in the dissolution and destruction of the embryo *in vivo* has also to be determined.

Summary.

1. *Mf. bancrofti*, kept alive *in vitro* for about fifty days, showed no change in morphology or growth.

2. Ex-sheathing is partly a mechanical process due to the entanglement of the sheath in leucocyte-fibrin masses derived from the blood. The embryo actively wriggles out after adhesion of the sheath.

Acknowledgments.

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ON CONTINUOUS BREEDING OF FLIES IN THE LABORATORY.

BY

D. N. ROY,

AND

L. B. SIDDONS.

(Financed by the Indian Research Fund Association.)

(From the Department of Medical Entomology, School of Tropical Medicine, Calcutta.)

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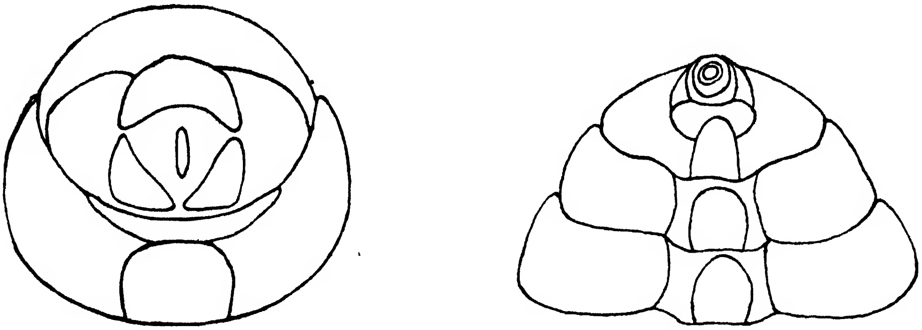
REARING flies in large numbers in the laboratory throughout the year where they may be required for physiological, biological, genetical, bacteriological and other research purposes is an important matter, and so simple and efficient methods of doing this should be readily available. Such studies are particularly important when flies of known age are needed. Methods of rearing flies have already been described by Glaser (1924, 1927), Grady (1928), Murdoch and Smart (1931), Hockenyos (1931) and Richardson (1932) but, as the species of *Muscoidea* encountered in India are different from those found in America and as the conditions prevailing in this country are also very different, we think it desirable to give a short summary of the process employed by us for the continuous rearing of the various species of local flies.

Wild flies of the desired species must be caught in food-stalls, bazaars, slaughter-house, meat-stalls, etc. Sometimes they are only available on carcasses. As the females are alone required, they should be separated from the males. The eyes are commonly approximated in male flies but this condition is not a reliable guide unless the worker is familiar with both sexes of the species. For sex determination it is necessary to examine the last visible segment. In females it is simple in contour, tapering towards the anus. Moreover, females caught in the

wild state will usually be found to be gravid, the abdomen distended with eggs and showing opaque white ventrally. In males the last segment is clearly modified to form part of the massive and complex genitalia and it is frequently of a different colour from the rest of the abdomen (*see* Text-figure).

TEXT-FIGURE.



MALE. FEMALE.

Ventral view of the last segment of the abdomen of *Chrysomyia megacephala.*

Gravid females are selected and confined individually in ordinary lamp chimneys, the tops of which are closed by gauze or mosquito-netting stuck down with plasticine. A thoroughly wet, but not dripping, wad of cotton-wool is placed on top, allowing communication with the outside air. This is the source of the fly's water-supply and must be replenished at least twice a day during the hot season. The chimney is placed upright in a close-fitting enamel dish containing the appropriate nidus (*see* Table). Carbohydrate must be provided for food, preferably as sugar or 'gur', either under the cotton-wool or in the receptacle containing the nidus. A small quantity of the nidus, e.g. horse manure, if *Musca vicina* is being cultured, is placed in the pan and when the eggs have been deposited, this is transferred to a larger quantity of the manure contained in another steep-sided pan or dish, about 12 inches in diameter, provided with a layer of sand at the bottom, half an inch in depth.

No reliability can be attached to the absence of contamination by flies in horse-dung supposed to have been collected soon after it has been passed. On several occasions we came across larvæ when the manure was allowed to stand for a few days. Eggs and small larvæ are difficult to detect.

Heating the manure is therefore essential in order to ensure it being free from other eggs and larvæ. It should be properly broken up and mixed with a little water and heated in a closed receptacle just short of the boiling point. It is then allowed to cool, care being taken not to remove the lid and thereby expose the manure to air.

TABLE.

Appropriate nidus for different flies.

Species of fly.	Egg-laying medium.	Food.
1. *C. rufifacies*	Meat	Meat-juice, sugar and water.
2. *Synthesiomyia nudiseta* ..	,,	
3. *C. megacephala*	,,	
4. *S. dux*	,,	
5. *S. ruficornis*	,,	
6. *M. vicina*	Horse-dung	
7. *M. nebulo* .. .	,,	
8. *M. sorbens*	Stool	
9. *M. vetustissima*	,,	
10. *M. yerburyi*	Horse-dung	
11. *Lucilia cuprina*	Meat	

When the nidus is meat, it should not be boiled, as fly larvæ do not thrive on boiled meat; slightly putrid meat is preferable. Care must be taken to see that the meat is not already contaminated by the eggs or maggots of other flies. Flesh-flies in particular may deposit their maggots through the meshes of the gauze and hence closed glass-topped cages are better for rearing these flies. Small sized mosquito-feeding cages (6″ × 9″ × 6″) are very useful for this purpose.

When the eggs (or maggots in the case of *Sarcophaga*) have been deposited, the whole is transferred to a larger quantity of the same medium contained in another steep-sided dish, about 12 inches in diameter, provided with a layer of dry sand for pupation, and the dish, suitably labelled, is kept in a fly-proof box or cage. The pupæ are later removed to a separate cage for breeding out the adults. The pupæ of *Synthesiomyia nudiseta*, which is known to cause secondary myiasis, are enclosed in cocoons which may be completely invested with sand grains and hence missed.

Flies mate readily in captivity, and as the sex ratio is roughly 1 : 1 except in *Chrysomyia ruffacies* Macq., there should be no difficulty in breeding them. In the case of *Chrysomyia rufifacies* it will be necessary to breed from several wild females, as our experience shows that the progeny of a single female are all of the same sex (Roy and Siddons, 1939).

During the summer season in Calcutta when conditions are most favourable for fly-breeding, flies will oviposit four to six days after emergence and the second complement of eggs will mature in the same period after the first batch has been deposited. During the winter when the atmosphere is comparatively colder and drier, this period is considerably extended even up to a week, and the period of development from egg to adult is also prolonged. In places in India where the winter is more severe, it may be necessary to place the breeding cage where it can be artificially heated to 75°F. to 80°F. and plenty of moisture should be provided.

It must be pointed out that the essential points in the details of any method connected with the continuous breeding of flies are :—

(1) Hydrolysed protein diet for nutrition of the ova.

(2) Avoidance of excessive or too little moisture in the culture medium.

(3) Presence of abundant air which is essential for the growth of the larvæ ; for this purpose the horse manure should be lightly placed in the pot and must not be pressed down with the finger.

(4) Abundance of food in the medium.

One other precaution is necessary: the protection of the pupæ from the visitations of parasitic *Hymenoptera*. A small species (*Spalangia* sp.) may gain access through the meshes of the wire-gauze of the fly-proof box and parasitize pupæ of *Musca* spp.

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